Microscale characterisation of a manufacturing route for lentiviral vectors

A thesis submitted to University College London (UCL)

for the degree of DOCTOR OF ENGINEERING

by

Heather Miranda Guy

January 2015

The Advanced Centre for Biochemical Engineering

Department of Biochemical Engineering

University College London

Torrington Place

London WC1E 7JE

I, Heather Miranda Guy, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Lentiviral vectors used in clinical trials are currently produced by transient transfection of adherent human embryonic kidney (HEK)293(T) cells. However, this approach is not scalable and for commercialisation the development of alternative strategies based on suspension-adapted producer cell lines, that have all genes for vector production stably integrated, is desired. To assist progress in this area, the aim of this thesis was to establish a microscale cell culture platform that enables key bioprocess design data to be acquired rapidly and cost-effectively. ProSavin®, an equine infectious anaemia virus (EIAV)-derived lentiviral vector developed for the treatment of Parkinson's disease (Palfi *et al.*, 2014) was used as a model system. First, the suitability of a shaken 24-well plate system for the suspension culture of HEK293T-derived producer cells was established. This system was shown to support equivalent cell growth and ProSavin® titres to conventional shake flasks while providing substantially greater opportunity for parallelisation. Second, the utility of the microscale platform when combined with statistical Design of Experiments (DoE) techniques for optimising titres and informing the design of a scale-up strategy was demonstrated. An initial screening experiment identified three parameters as having a critical influence on ProSavin® titres, which were post-induction period, liquid fill volume and concentration of doxycycline (inducer compound). Subsequent optimisation experiments defined operating ranges for these parameters. Third, the insights obtained during the microwell investigations were shown to aid successful scale-up of the ProSavin® process to a single-use 2 L WAVE bioreactor. Fourth, with a view to informing further improvements in process design, the half-life of ProSavin® and other EIAV-based lentiviral vectors was determined, and approaches to moderate the rate of decay during upstream processing trialled. Overall, it was concluded that the microwell platform may be viewed as an effective tool for future use in the development of lentiviral vector bioprocesses.

Acknowledgements

I am grateful to my supervisors Tarit Mukhopadhyay and Gary Lye at UCL, and Kyriacos Mitrophanous at Oxford BioMedica, for their patience and guidance throughout my studies. At Oxford BioMedica I would like to thank colleagues in Research, PAR Group and the laboratory management team for their help and advice; I am particularly grateful to Kirstie Pemberton (*née* Freail), Carol Knevelman, Hanna Pardo, Linda Barrett and Julia Sutton. I would like to thank my departmental colleagues at UCL, particularly John Betts, for the encouragement, laughter and numerous cups of tea. Lastly, I would also like to thank my family and, most of all, Danny, for their unwavering support.

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Nomenclature

AADC	aromatic amino acid decarboxylase
ADA	adenosine deaminase
ADA$^-$ SCID	severe combined immunodeficiency caused by ADA deficiency
AdjR^2	adjusted R^2
ALD	adrenoleukodystrophy
ANOVA	analysis of variance
ATP	adenosine triphosphate
BIV	bovine immunodeficiency virus
CA	capsid
CAD	Computer Aided Design
CCC	central composite circumscribed
CCF	central composite face-centred
CH1	GTP-cyclohydrolase 1
CHO	Chinese hamster ovary (cell line)
CAEV	caprine arthritis-encephalitis virus
CMV	cytomegalovirus
CMVp	cytomegalovirus promoter
CPP	critical processing parameter
CQA	critical quality attribute
CSO	Chief Scientific Officer
CV	coefficient of variation
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DoE	Design of Experiments
dox	doxycycline
DU	dUTPase
eGFP	enhanced green fluorescent protein
EIAV	equine infectious anaemia virus

EMA	European Medicines Agency
Env	envelope (viral structural protein)
FCS	foetal calf serum
FDA	Food and Drug Administration
FIV	feline immunodeficiency virus
Fr	Froude number (see Section 2.5 for calculation)
Gag	group specific antigen (lentiviral structural protein)
GFP	green fluorescent protein
GMP	Good Manufacturing Practice
HEK293	human embryonic kidney 293 cell line
HEK293T	modified HEK293 cell line expressing the SV40 large T antigen
HIV	human immunodeficiency virus
ICH	The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IN	integrase
IRES	internal ribosome entry site
k_La	oxygen mass transfer coefficient
LacZ	gene for β-galactosidase
LOQ	limit of quantification
LPL	lipoprotein lipase
LPLD	lipoprotein lipase deficiency
LTR	long terminal repeat
MA	matrix
MLV	murine leukaemia virus
MMV	ovine maedi-visna virus
mRNA	messenger ribonucleic acid
NC	nucleocapsid
Nef	negative factor (lentiviral accessory protein)
OFAT	one-factor-at-a-time
ORF	open reading frame
OTC	ornithine transcarbamylase

pA	polyadenylation signal
PAT	process analytical technology
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
PERT	product enhanced reverse transcriptase
Ph	Phase number (see Section 2.5 for calculation)
P:I ratio	particle:infectivity ratio (RNA copy number / titre)
PIC	pre-integration complex
Pol	polymerase (lentiviral structural protein)
PPQ	process performance qualification
PR	protease
PredR^2	predicted R^2
PS46.2	stable ProSavin® producer cell line derived from HEK293T
P/V	energy dissipation rate (power per unit volume)
QbD	Quality by Design
qPCR	quantitative polymerase chain reaction
R	repeated
R^2	coefficient of determination
RCL	replication competent lentivirus
Re	Reynolds number (see Section 2.5 for calculation)
Rev	regulatory viral protein (lentiviral accessory protein)
RNA	ribonucleic acid
rpm	revolutions per minute, unless otherwise stated
RT	reverse transcriptase
RT qPCR	reverse transcriptase quantitative polymerase chain reaction
SCID	severe combined immunodeficiency
SCID-X1	X-linked severe combined immunodeficiency
SD	standard deviation
SIN	self-inactivating
SIV	simian immunodeficiency virus

SU	surface envelope glycoprotein
SV40	simian vacuolating virus 40
Tat	trans-activator of transcription (lentiviral accessory protein)
tet	tetracycline
TetR	tetracycline repressor
TH	tyrosine hydroxylase
TM	transmembrane envelope proteins
TU	transducing units
Vif	viral infectivity factor (lentiviral accessory protein)
Vpr	viral protein r (lentiviral accessory protein)
Vpu	viral protein u (lentiviral accessory protein)
Vpx	viral protein x (lentiviral accessory protein)
VSV-G	vesicular stomatitis virus G glycoprotein
WAS	Wiskott-Aldrich Syndrome
WPRE	woodchuck hepatitis virus posttranscriptional regulatory element
X-CGD	X-linked chronic granulomatous disease

Greek symbols

Ψ	viral packaging signal

1 Introduction

1.1 Thesis rationale and overview

In November 2012, a significant milestone was reached: Glybera became the first human gene therapy to receive marketing approval in the Western world (uniQure, 2012; Kastelein *et al.*, 2013). Other gene therapies are soon expected to follow suit, offering exciting prospects for the treatment of various diseases. Gene therapies that use lentiviral vectors to mediate gene delivery have shown particular promise, with lentiviral vectors being employed in over sixty clinical trials to date (Segura *et al.*, 2013). However, as these clinical programmes advance towards commercialisation, a primary concern is whether existing manufacturing practices are capable of generating sufficient quantities of material to meet the expected demands. The majority of lentiviral vectors used in clinical trials are currently produced by transient transfection of adherent human embryonic kidney (HEK)293(T) cells, however this method is problematic to scale-up (Schweizer and Merten, 2010). The development of a simple, scalable manufacturing route based on the suspension culture of stable producer cell lines is highly desirable. Despite this, few studies have explored large-scale suspension-based production strategies for lentiviral vectors (Segura *et al.*, 2007; Broussau *et al.*, 2008; Ansorge *et al.*, 2009; Witting *et al.*, 2012).

To aid the development of industrially viable bioprocesses for lentiviral vectors, a better understanding of the factors influencing yields from suspension cultures of HEK-derived producer cells is required. As a variety of parameters may impact on titres, numerous experiments are necessary to characterise their various effects. A rational approach combining microscale bioprocessing and Design of Experiments (DoE) techniques could simplify this process. Microscale bioprocessing facilitates numerous culture conditions to be evaluated quickly and simultaneously, and at low-cost (Kumar *et al.*, 2004; Micheletti and Lye, 2006; Lye *et al.*, 2009; Marques *et al.*, 2010; Neubauer *et al.*, 2013), while DoE enables efficient characterisation of the effects of multiple parameters through a limited number of logically linked experiments (Czitrom, 1999; Tye, 2004; Anderson and Whitcomb, 2007; Eriksson *et al.*, 2008). The value of such an approach has been demonstrated for the optimisation of protein expression from microbial cultures (Islam *et al.*, 2007; Holmes *et al.*, 2009). It was recently proposed that DoE techniques could aid the development of lentiviral vector production

systems, leading to more efficient bioprocesses and better characterised products (Segura *et al.*, 2013). However, there are no published studies that have employed this methodology, or tested the value of a suspension-based microscale system, for lentiviral vector process development to date. Thus, the aim of this work is to establish a microwell experimental platform for the suspension culture of lentiviral vector producer cells, which can be utilised in conjunction with DoE techniques for the rapid, early generation of critical bioprocess design data. The approach will be demonstrated and published on using ProSavin®, a lentiviral vector engineered for treatment of Parkinson's disease that has successfully completed a Phase I/II clinical trial (Mitrophanous *et al.*, 1999; Azzouz *et al.*, 2002; Jarraya *et al.*, 2009; Stewart *et al.*, 2009; Stewart *et al.*, 2011; Palfi *et al.*, 2014).

1.2 Introduction to gene therapy

This section provides a brief introduction to gene therapy, through (i) outlining some of the major milestones and setbacks that have defined progress in the field, (ii) summarising the current status of ongoing gene therapy clinical trials, and (iii) considering some of the remaining challenges.

1.2.1 An overview of gene therapy

'Gene therapy' may be broadly defined as the introduction of therapeutic genes into target cells in order to treat disease (Escors and Breckpot, 2010). However, it is important to note that applications of gene therapy are far-reaching, and the Food and Drug Administration (FDA) and European Medicines Agency (EMA) have published more lengthy definitions (see FDA, 2006; EMA, 2009) that strive to capture recent innovations within this field. Importantly, a distinction exists between somatic gene therapy, in which the genetic change is restricted to the individual patient, and germ line gene therapy, in which the genetic change is heritable and thus passed on to the patient's offspring; the latter is not permitted by current legislation (Wirth *et al.*, 2013).

From the 1960s to the 1990s gene therapy progressed from conception to clinical evaluation; an evolution largely catalysed by the advent of recombinant DNA techniques and the development of virus-based gene delivery vectors (for an overview see Friedmann, 1992). In 1989, the first Federally approved gene transfer study using human patients took place (Rosenberg *et al.*,

1990). A marker gene (conferring neomycin resistance) was introduced into autologous tumour-infiltrating lymphocytes *in vitro* using a retroviral vector; the modified lymphocytes were then reintroduced into patients with advanced melanoma and their distribution monitored over time (Rosenberg *et al.*, 1990). Although this study did not have a therapeutic aim, it critically demonstrated the potential of retroviral vectors for gene transfer (Edelstein *et al.*, 2004). In 1990, the first Federally approved clinical trial using a therapeutic gene commenced, in which two children with severe combined immunodeficiency (SCID) caused by adenosine deaminase (ADA)-deficiency (ADA^- SCID) received autologous T cells that had been transduced *in vitro* by a retroviral vector carrying a functional ADA gene (Blaese and Anderson, 1990; Blaese *et al.*, 1995). Although this early attempt at gene therapy for treatment of ADA^- SCID did not correct the immunodeficiency, the viability and safety of the procedure was demonstrated and transduced T cells persisted up to 15 - 20 years later (Fischer *et al.*, 2010). In the decade succeeding these pioneering studies (1989 - 1999) close to 500 gene therapy clinical trials were approved worldwide, including an unprecedented 116 trials in the year 1999 alone (Ginn *et al.*, 2013). These figures are indicative of a rapidly expanding, thriving field. Unfortunately, the advances made during this period were cast into shadow when, in 1999, Jesse Gelsinger became the first clinical trial patient to die as a direct consequence of gene therapy (Hollon, 2000). Gelsinger, a teenager with partial ornithine transcarbamylase (OTC) deficiency, died from vector-related toxicity shortly after receiving an adenoviral vector carrying the OTC gene (Hollon, 2000; Check, 2005). A five-year investigation by the US Department of Justice revealed that several mistakes had been made during the study, including failure to inform the Recombinant DNA Advisory Committee of an alteration in the protocol and failure to report a serious adverse reaction to therapy (Hollon, 2000; Check, 2005; Couzin and Kaiser, 2005; Sheridan, 2011). The Gelsinger case was significant, as it illuminated widespread underreporting of adverse events in gene therapy trials in general (Teichler Zallen, 2000; Sheridan, 2011); the FDA and National Institutes of Health responded by launching new initiatives to provide greater protection for patients enrolled in gene therapy trials and to reinstate public trust in gene therapy research (McCarthy, 2000). In the years during and succeeding the Gelsinger case, the field of gene therapy suffered a further blow as several instances of insertional mutagenesis following (gamma)retroviral mediated gene therapy were reported. In two trials for X-linked SCID (SCID-X1), autologous haematopoietic cells were

transduced *in vitro* using a vector encoding the interleukin 2 receptor-γ (*IL2RG*) gene and re-introduced into patients (twenty in total by the end of the study), however five patients went on to develop a T cell leukemic disease due to the activation of proto-oncogenes as a consequence of provirus integration (Hacein-Bey-Abina *et al.*, 2008; Howe *et al.*, 2008; Fischer *et al.*, 2010; Hacein-Bey-Abina *et al.*, 2010). Insertional mutagenesis also led to the development of T cell leukaemia in one out of ten patients in trials targeting Wiskott-Aldrich Syndrome (WAS) (Galy and Thrasher, 2011) and to the development of clonal myeloproliferation in three out of twelve patients in clinical trials addressing X-linked chronic granulomatous disease (X-CGD) (Grez *et al.*, 2011). The toxicity observed in the Gelsinger case and instances of insertional mutagenesis observed in the SCID-X1, WAS and X-CGD trials highlighted serious safety concerns associated with the use of early generation adenoviral and retroviral vectors. This has led to the development of better investigative and analytical tools, such as animal models of insertional mutagenesis (Montini *et al.*, 2009) and high throughput insertion site analysis techniques (Schmidt *et al.*, 2007) that have been the basis for the more recent successes in gene therapy.

1.2.2 The current status of gene therapy

By June 2012, 1843 gene therapy clinical trials had been approved worldwide (Ginn *et al.*, 2013). Using the data presented in Ginn *et al.* (2013), the geographical distribution, disease targets, gene types, delivery methods, and clinical phase of these trials are summarised here. Note that the data presented by Ginn *et al.* (2013) is also available as a searchable database that is updated periodically on *The Journal of Gene Medicine* Gene Therapy Clinical Trials Worldwide website (http://www.wiley.co.uk/genmed/clinical/).

The majority of gene therapy trials (63.7 %) have been performed in the US, followed by the UK (11 %), Germany (4.4 %), France (2.9 %) and Switzerland (2.7 %) (Ginn *et al.*, 2013). Cancer is the second leading cause of death in the West (Ortiz *et al.*, 2012), and around two thirds of gene therapy trials worldwide have targeted cancer diseases (Table 1.1; Ginn *et al.* (2013)).

Table 1.1 Indications addressed in gene therapy clinical trials from 1989 to June 2012. Data was derived from Ginn *et al.* (2013).

Indications	Gene therapy clinical trials	
	%	Number
Cancer diseases	64.4	1186
Monogenic diseases	8.7	161
Cardiovascular diseases	8.4	155
Infectious diseases	8.0	147
Neurological diseases	2.0	36
Ocular diseases	1.5	28
Inflammatory diseases	0.7	13
Other diseases	1.4	25
Gene marking	2.7	50
Healthy volunteers	2.3	42
Total		1843

The gene types most commonly transferred in gene therapy trials were antigens (e.g. viral vector-mediated transfer of tumour antigens to intensify the immune response in a tumour-bearing patient (Edelstein *et al.*, 2007)), cytokines, tumour suppressors and suicide enzymes, which are principally employed to tackle cancer (the category most targeted by gene therapy), and together comprised 55.3 % of all trials (Ginn *et al.*, 2013). The majority of trials employed modified viruses ('viral vectors') to deliver the genetic material into target cells (Table 1.2). Viruses have naturally evolved effective mechanisms for infecting various cell types, thus delivery vectors exploiting this capacity are more efficient than alternative non-viral techniques (Warnock *et al.*, 2006). Adenoviral and retroviral vectors have been most commonly utilised (Table 1.2), although since the Gelsinger case and the incidences of leukaemia in SCID-X1 patients (see Section 1.2.1) there has been a movement away from these vector types (Sheridan, 2011). To January 2004, 26 % of all trials employed adenoviral vectors and 28 % used retroviral vectors (Edelstein *et al.*, 2004), but by June 2012, these values had decreased to 23.3 % and 19.7 %, respectively (Ginn *et al.*, 2013). However, adenoviral and retroviral vectors continue to be utilised today, and published data indicates that between July 2007 and June 2012, 107 adenovirus-based trials and 65 retrovirus-based trials were approved (Edelstein *et al.*, 2007; Ginn *et al.*, 2013). Adenoviral and retroviral vectors with improved safety profiles are in development (Vetrini and Ng, 2010; Sadelain *et al.*, 2012), and alternative viral and non-viral delivery methods are increasingly being explored (Table 1.2).

Table 1.2 Gene delivery methods employed in gene therapy clinical trials from 1989 to June 2012. Data was derived from Ginn *et al.* (2013).

Gene delivery method	Gene therapy clinical trials	
	%	Number
Adenovirus	23.3	438
Retrovirus	19.7	370
Naked/Plasmid DNA	18.3	345
Vaccinia virus	7.9	148
Lipofection	5.9	111
Poxvirus	5.0	95
Adeno-associated virus	4.9	92
Herpes simples virus	3.1	59
Lentivirus	2.9	55
Other categories	5.6	105
Unknown	3.4	64
Total		1882

The majority (78.6 %) of approved gene therapy clinical trials are in Phase I or I/II, less (16.7 %) are in Phase II, and very few (4.5 %) are in Phase II/III or III (Ginn *et al.*, 2013). This pattern is not unexpected, as attrition rates are generally high within the pharmaceutical sector- on average just 11 % of new therapies tested in humans ever reach registration (Kola and Landis, 2004). For cancer, the leading indication being addressed by gene therapy (Table 1.1), the success rate is even lower, with just 5 % of new treatments achieving registration (Kola and Landis, 2004). Added to this is the fact that gene therapy is an emerging field, with the regulatory framework, and also the scientific tools to measure safety and efficacy, still being developed. Nonetheless, to date, two gene therapy products have been granted marketing authorisation. In October 2003, Shenzhen SiBiono GenTech (China) received a license from the State Food and Drug Administration of China for Gendicine, an adenoviral vector carrying the p53 tumour suppressor gene used for treatment of patients with head and neck squamous cell carcinoma (Pearson *et al.*, 2004). The only gene therapy currently licensed in the West is Glybera (or alipogene tiparvovec), for which uniQure (The Netherlands) received EMA marketing approval in November 2012 (Bryant *et al.*, 2013; Kastelein *et al.*, 2013). Gybera is an adeno-associated viral vector containing a functional human lipoprotein lipase (LPL) gene, engineered for treatment of patients with LPL deficiency (LPLD) (Bryant *et al.*, 2013; Kastelein *et al.*, 2013). As other gene therapies approach marketing authorisation, fresh challenges are emerging within the sector as discussed below.

1.2.3 Future challenges for gene therapy

Work to improve the safety of gene therapy and refinements to existing vector delivery systems are ongoing. For example, to diminish the incidence and severity of immune reactions to adenoviral vectors, helper-dependent adenoviral vectors, that are devoid of viral coding sequences, are in development (Vetrini and Ng, 2010). In addition, to reduce the risk of insertional mutagenesis, self-inactivating (SIN) retroviral vectors, in which all enhancer and promoter elements have been deleted from the U3 region of the long terminal repeat (LTR), have been described (Modlich *et al.*, 2006). However, although for some gene therapies ongoing improvement of the vector delivery system is essential, for others, adequate evidence of safety and therapeutic efficacy has been gathered and soon marketing authorisation will be sought. The approval granted by the EMA for Glybera was a landmark case, and has opened the door to other organisations seeking to register gene therapies in the West. As these therapies approach commercialisation, however, an immediate concern is whether current manufacturing practices are suitable for large-scale industrial applications. It is a concern experts agree has received inadequate attention and investment to date (Sheridan, 2011; Dolgin, 2012).

The development of a scalable bioprocess that is compliant with Good Manufacturing Practice (GMP) standards is recognised as major a hurdle to gene therapy commercialisation (Denèfle, 2011). James Wilson (Perelman School of Medicine, University of Pennsylvania), recently acknowledged “the rate-limiting step in most of these [gene therapy] programmes is a scalable, commercial-grade process”, while Fulvio Mavilio, scientific director of Généthon (a not-for-profit gene therapy organisation based in France), described the state-of-the-art as “primitive” and stated “we’re not talking about robust processes that can be scaled up for manufacturing. Not at all” (Dolgin, 2012). A fundamental problem is that production systems are typically based on adherent cell culture, for example adenoviral (Dormond and Kamen, 2011), retroviral (Cruz *et al.*, 2011), adeno-associated viral (Chen, 2011) and lentiviral (Schweizer and Merten, 2010) vectors are all commonly generated from human-derived cell lines grown in monolayers. Sander van Deventer, speaking as Chief Scientific Officer (CSO) of Amsterdam Molecular Therapeutics in The Netherlands (which developed Glybera but has since been renamed as uniQure) commented “you need to have a soluble cell system” as adherent cell systems cannot

be scaled-up (Sheridan, 2011). This point was echoed by Stuart Naylor, CSO of gene therapy company Oxford BioMedica (UK), who noted that scale-up by the addition of extra cell culture vessels (i.e. a 'scale-out' approach) is not feasible, as this leads to a disproportionately large quantity of the final product being consumed by quality testing (Sheridan, 2011). Using suspension-grown cells to produce gene therapy vectors mitigates this problem, as three dimensional scale-up into multi-litre bioreactors is then feasible. Interestingly, Glybera is manufactured using suspension-cultured insect cells (Dolgin, 2012; Bryant *et al.*, 2013) even though, for this particular gene therapy, process scalability was never a major hurdle to commercialisation as LPLD affects fewer than 1,000 people across Europe (Dolgin, 2012). The use of insect cells was possible in this case because a non-enveloped adeno-associated viral vector was employed - for enveloped vectors, such as those based on retroviruses or lentiviruses, this approach is more problematic (Fernandes *et al.*, 2013). Thus, for other gene therapies approaching registration, which target more prevalent indications, the development of scalable GMP-compliant industrial processes remains a crucial challenge.

Further recognised obstacles to gene therapy commercialisation relate to the purification and final formulation of the product (Denèfle, 2011). In particular, impurities left over from the upstream process, such as animal serum, contaminating DNA and host cell proteins can present a concern (Segura *et al.*, 2006; Jolly and Aguilar-Cordova, 2008). Where possible, the best solution is to avoid the introduction of such components in the first place. For example, switching to chemically-defined media and stable producer cell lines would eliminate anxieties relating to the presence of animal serum and plasmid DNA originating from cell transfections. Host cell-derived contaminants, however, must be removed later in the process, and this can prove problematic. For example, John Gray, director of the Vector Development and Production Shared Resource at St Jude Children's Research Hospital (Tennessee) recently commented that fragments of host cell DNA commonly end up *inside* viral vectors, meaning they cannot be eradicated from the final product (Dolgin, 2012). It is possible some of these fragments may be necessary for the proper formation and functioning of viral vectors. It is currently unknown whether such genetic impurities are harmless or if they have in fact contributed to the inflammatory side effects observed in gene therapy trials to date (Dolgin, 2012). Another difficulty of virus-based gene delivery systems is that transduction-competent

particles typically comprise a small proportion of total particles in a given preparation (Kay *et al.*, 2001). For example, preparations of lentiviral and adeno-associated viral vectors can contain around 10,000 non-functional particles for each single functional particle (Jolly and Aguilar-Cordova, 2008; Stewart *et al.*, 2009). Of particular concern are 'empty' particles, which do not contain vector RNA, as they compete with functional particles for entry to target cells and also may provoke unwanted immune reactions (Zhao *et al.*, 2008; Sheridan, 2011). Removal of empty particles downstream is thus highly desirable, however they are particularly troublesome to quantitate and isolate due to their inherent similarity in size and protein composition to complete particles, although some success has been achieved using anion exchange or size exclusion chromatography techniques (Qu *et al.*, 2007; Zhao *et al.*, 2008). Lastly, in addition to purification, significant concentration of gene therapy products is typically required to attain clinical efficacy. Routine doses of gene therapy products are forecast to be within the range of 10^8 to 10^{15} viral particles per kg of bodyweight (Denèfle, 2011). For Glybera, the dose is 1 x 10^{12} genome copies per kg of bodyweight, which is achievable as the finished product contains 3 x 10^{12} genome copies per mL; a 60 kg patient, for example, would therefore receive 20 mL of finished product (EMA, 2012). When concentrating gene therapies for clinical administration, minimising the co-concentration of unwanted contaminants while maintaining product integrity remains a common challenge (Morenweiser, 2005). To sum-up, defining robust, commercially-viable manufacturing routes for gene therapy products is an area requiring significant attention.

1.3 Gene therapy using lentiviral vectors

Having provided a broad introduction to gene therapy in Section 1.2, the specific application of lentiviral vectors is examined here. To June 2012, lentiviral vectors had been employed in around 2.9 % (n = 55) of gene therapy clinical trials (Ginn *et al.*, 2013). In addition, recent research has demonstrated a more favourable integration pattern for lentiviral vectors as compared to conventional retroviral vectors (Dropulić (2011), and references therein), thus lentiviral vectors are becoming of increasing interest (Sheridan, 2011). Briefly outlined below is the general biology of lentiviruses, followed by an overview of lentivirus-based gene delivery systems. The section concludes by introducing ProSavin®, the lentiviral vector utilised as a model system throughout this work.

1.3.1 General characteristics of lentiviruses

Lentiviridae form a subfamily within the Retroviridae family of viruses, which also includes the Oncornaviridae and Spumaviridae; the groupings broadly reflect the nature of the pathogenesis experienced by the host (Wagner *et al.*, 2008). It is thus important to clarify that the term 'retroviral vectors' used within this thesis refers exclusively to vectors derived from oncornaviruses ('lentiviral vectors' obviously relates only to lentivirus-based vectors). Although several gene therapy vectors based on spumaviruses are in development (e.g., Horino *et al.*, 2013 and references therein), none have yet progressed to human clinical trials and this group will not be discussed further in this thesis.

Lentiviruses are pathogens of humans and animals (Table 1.3), causing diseases that are characterised by a long incubation period (*lenti* meaning 'slow' in Latin), are persistent despite strong immune responses, affect multiple organs and invariably prove fatal (Haase, 1986; Clements and Zink, 1996). Although equine infectious anaemia virus (EIAV) was the first lentivirus to be discovered (Table 1.3), human immunodeficiency virus (HIV)-1 has been most studied due to its critical impact on human health. Transmission of lentiviruses between individuals occurs solely by the exchange of bodily fluid, and EIAV is the only known lentivirus to be spread via an insect vector (Narayan and Clements, 1989). Most lentiviruses infect macrophages, and all lentiviruses can infect non-dividing cells (Wagner *et al.*, 2008), a feature that spurred the development of lentivirus-based vectors for gene therapy, as conventional oncornavirus-based vectors lack this capacity (Naldini *et al.*, 1996b).

Table 1.3 Common lentiviruses and their hosts, with a reference to their discovery or isolation. Information derived from Coffin *et al.* (1997) and Wagner *et al.* (2008).

Lentivirus	Host	Discovery/Isolation
Equine infectious anaemia virus (EIAV)	Horse	(Vallée and Carré, 1904)
Ovine maedi-visna virus (MMV)	Sheep	(Sigurdsson, 1954)
Bovine immunodeficiency virus (BIV)	Cattle	(Van der Maaten *et al.*, 1972)
Caprine arthritis-encephalitis virus (CAEV)	Goat	(Cork *et al.*, 1974)
Human immunodeficiency virus 1 (HIV-1)	Human	(Barré-Sinoussi *et al.*, 1983)
Simian immunodeficiency virus (SIV)	Monkey	(Letvin *et al.*, 1985)
Human immunodeficiency virus 2 (HIV-2)	Human	(Clavel *et al.*, 1986)
Feline immunodeficiency virus (FIV)	Cat	(Pedersen *et al.*, 1987)

Lentiviral particles contain two copies of positive-sense strand RNA genome, which are complexed with nucleocapsid (NC) proteins and enclosed within a capsid (CA) that also contains the protease (PR), integrase (IN) and reverse transcriptase (RT) enzymes that play fundamental roles in the lentiviral life cycle (Rodrigues *et al*., 2011; Wagner *et al*., 2008; Figures 1.1 and 1.2). A layer of matrix proteins (MA) outside the CA interacts with the envelope (a lipid bilayer derived from the host cell) (Rodrigues *et al.*, 2011). Viral envelope (Env) proteins are anchored within the lipid membrane by a transmembrane (TM) subunit, while the surface (SU) subunit interacts with cellular receptors (Rodrigues *et al.*, 2011).

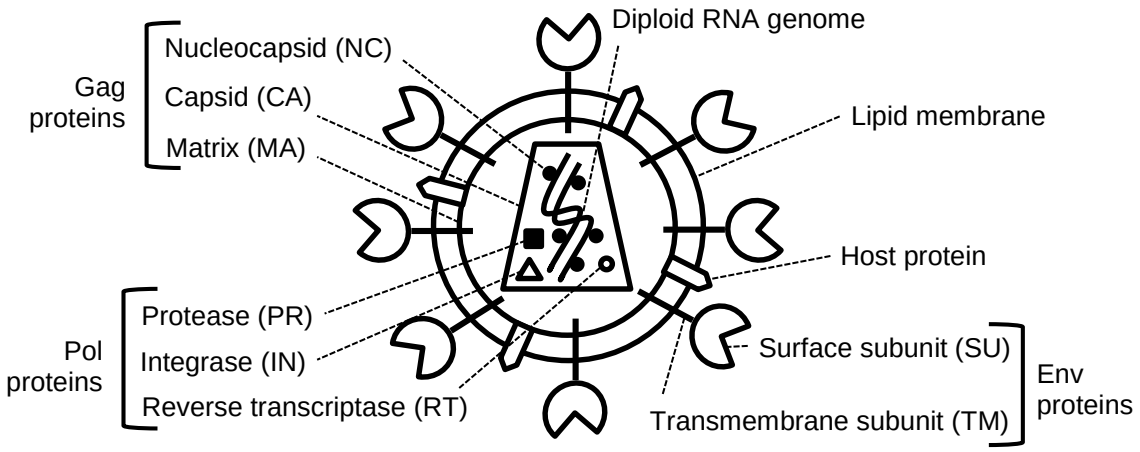


Figure 1.1 Schematic representation of lentivirus particle. Gag (group specific antigen), Pol (polymerase) and Env (envelope) proteins are indicated. Note: actual diameter of a lentivirus particle is approximately 119 nm (Wagner *et al.*, 2008). Figure is adapted from illustrations presented in Segura *et al.* (2006) and Wagner *et al.* (2008).

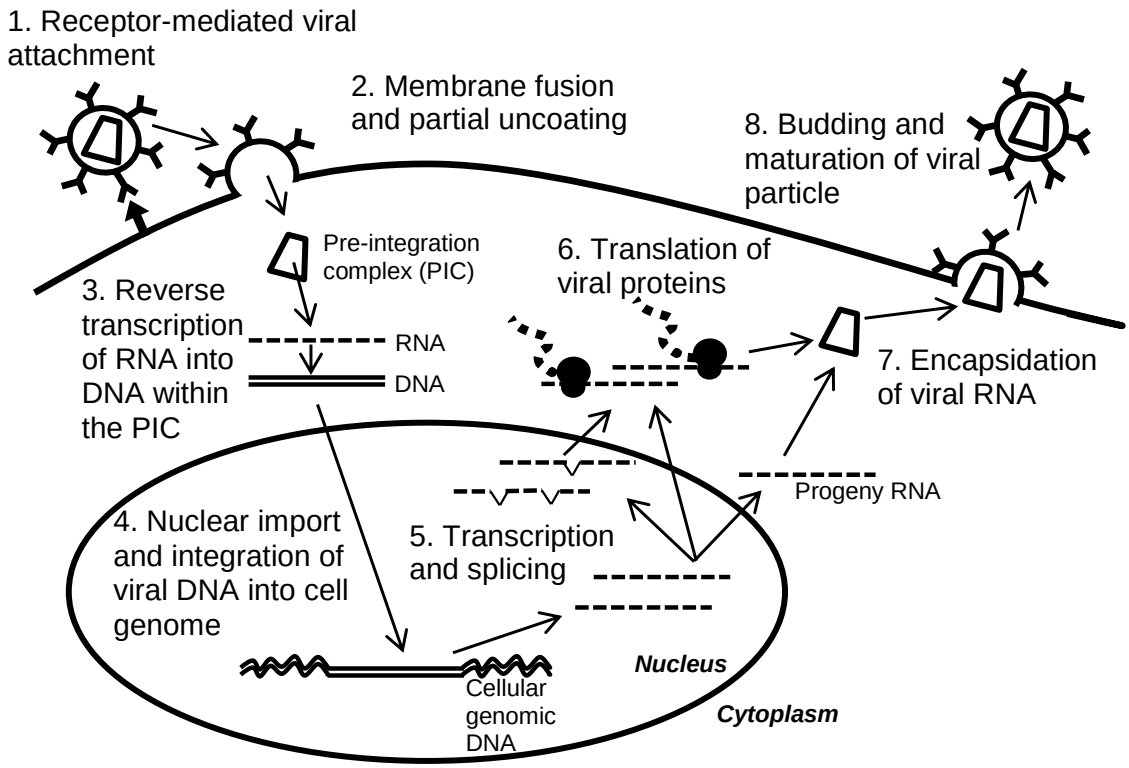


Figure 1.2 Schematic representation of the lentivirus life cycle. Note that the reverse transcriptase (RT) enzyme mediates reverse transcription of lentiviral RNA into DNA (step 3.); integrase (IN) facilitates the integration of proviral DNA into the cell genome (step 4.); and protease (PR) cleaves Gag and Gag-Pol during the assembly, budding and maturation of particles (steps 7. and 8.) (Wagner *et al.*, 2008; Rodrigues *et al.*, 2011). Figure is adapted from illustrations presented in Leroux *et al.* (2004) and Wagner *et al.* (2008).

The basic genetic organisation of lentiviruses is largely analogous to that of oncornaviruses. R (repeated) regions that contain transcriptional signals utilised in proviral DNA are located at both ends of the genome (Wagner *et al.*, 2008). Following the R sequence at the 5' end of genomic DNA is (i) a U5 non-coding *cis*-acting regulatory sequence (ii) a primer binding site where cellular transfer (t)RNA binds and acts as a primer for initiation of reverse transcription, and (iii) a leader sequence that comprises the genome packaging signals used in virus maturation and also splice donor signals that are important in creating spliced lentiviral messenger (m)RNAs (Wagner *et al.*, 2008). The 3' end of genomic DNA contains a polypurine tract that is essential for the generation of DNA from RNA, a U3 non-coding sequence and a second copy of the R sequence (Wagner *et al.*, 2008). Similar to oncornaviruses ('simple' retroviruses), lentiviruses encode the structural genes *gag*, *pol* and *env*; however, lentiviruses ('complex' retroviruses)

additionally express several accessory proteins that confer the unique life cycle presented in Figure 1.2 (Wagner *et al.*, 2008). An overview of lentiviral proteins and their functions is given in Table 1.4.

Table 1.4 Lentiviral proteins and their functions. Only proteins common to all lentiviruses and those that are primate-related are listed, as these have been best-studied. Lentiviruses known to express each given protein are indicated (for non-abbreviated names refer to Table 1.3). Information was derived from Federico (2003).

Protein	Function	Lentiviruses
Structural		
Group specific antigen (Gag)	Gag precursor proteins are cleaved to form the nucleocapsid, capsid and matrix of the viral particle.	All
Polymerase (Pol)	Gag-Pol precursor proteins are cleaved to generate viral protease, integrase and reverse transcriptase enzymes.	All
Envelope (Env)	The Env precursor protein is cleaved to create the viral surface and transmembrane envelope proteins.	All
Accessory		
Trans-activator of transcription (Tat)	Mediates elongation of viral transcripts.	All
Regulatory viral protein (Rev)	Facilitates the export of unspliced and partially spliced viral RNA from the cell nucleus into the cytoplasm.	All
Negative factor (Nef)	Promotes viral infectivity by mechanisms not yet fully understood.	HIV-1, HIV-2, SIV
Viral infectivity factor (Vif)	Counteracts a cellular inhibitor of viral replication.	All except EIAV
Viral protein r (Vpr)	Arrests cell cycle in G_2 phase. Aids migration of viral pre-integration complex towards cell nucleus.	HIV-1, HIV-2, SIV
Viral protein u (Vpu)	Forms ion channels in cell membrane to aid viral release.	HIV-1
Viral protein x (Vpx)	Aids migration of viral pre-integration complex towards cell nucleus.	HIV-2, SIV

1.3.2 The development of lentiviral vectors for gene therapy

As noted in Section 1.3.1, the capacity of lentiviruses to transduce non-diving cells provided impetus for their application as gene therapy vectors. Further successful attributes of lentiviral vectors, in common with conventional retroviral vectors, include their ability to integrate within the cellular genome and an absence of pre-existing immunity in the recipient (Ansorge *et al.*,

2010). Lentiviral vector development culminated in 1996 in the first description of a practical system by Naldini *et al.* (1996a), who achieved successful *in vivo* transduction of rat neurons using a HIV-1-derived vector system. Since this landmark study numerous lentiviral vectors have been developed, though the majority are still based on HIV-1, and the biosafety of these systems has improved over time with regard to minimising the risk of replication-competent lentivirus (RCL) formation (Binder and Dropulic, 2008; Ansorge *et al.*, 2010). The 'first-generation' vector system developed by Naldini *et al.* (1996a) expressed seven out of the nine wild-type HIV-1 genes (*vpu* and *env* were rendered defective), and vectors were generated by three-plasmid transient transfections. All HIV-1 *trans*-acting sequences (i.e those encoding viral proteins) were contained on one plasmid (the 'packaging plasmid'), while regulatory *cis*-acting elements were confined to a separate 'transfer plasmid', which also comprised the transgene (encoding a fluorescent marker); the third 'envelope' plasmid encoded either the murine leukaemia virus (MLV) amphotrophic envelope protein or the vesicular stomatitis virus envelope protein (VSV-G), for a broad tissue tropism (Naldini *et al.*, 1996b). Separation of vector constructs on to three plasmids is an important technique for the prevention of recombination events leading to RCL, however further steps to improve the biosafety of lentiviral vectors were necessary (Binder and Dropulic, 2008). Thus, second-generation vectors were developed, in which the virulence genes *env*, *nef, vif*, *vpr* and *vpu* were deleted (Zufferey *et al.*, 1997), and soon after third-generation vectors were established, in which the *tat* gene was also removed (offset by the insertion of a constitutive promoter upstream of the vector transgene) (Kim *et al.*, 1998) and *rev* was confined to a separate expression plasmid to that encoding *gag* and *pol* (Dull *et al.*, 1998). The latest generations of lentiviral vectors are also 'self-inactivating' (SIN), as they include a deletion in the U3 region of the LTR that is transferred to the 5' LTR during reverse transcription, preventing the generation of full-length vector RNA in target cells (Zufferey *et al.*, 1998). Finally, the discovery that inclusion of the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) could significantly enhance transgene expression (Zufferey *et al.*, 1999) was a critical development that helped advance lentiviral vectors to clinical application.

In the first clinical trial to utilise lentiviral vectors, initiated in 2003, patients with chronic HIV infection received autologous $CD4^+$ T cells that had been modified *ex vivo* by a HIV-1-derived vector encoding an antisense gene against the HIV envelope (VRX496^{TM}) (Manilla *et al.*, 2005;

Levine *et al.*, 2006). Up to January 2013, 62 lentiviral vector-based clinical trials had been undertaken (Segura *et al.* (2013); Table 1.5). Although gene therapy trials overall have mostly addressed cancer (Section 1.2.2), trials based on lentiviral vectors have largely targeted monogenic diseases (Table 1.5). This may be explained by the fact that, unlike non-integrating vectors (e.g. those derived from adenoviruses and adeno-associated viruses), lentiviral vectors can permanently integrate therapeutic genes within host cell DNA (Thomas *et al.*, 2003), thus they hold the potential to confer life-long disease correction. Since trials employing the VRX496^{TM} vector were initiated more than 10 years ago 65 HIV-positive patients have been treated and, encouragingly, no adverse events have been reported (McGarrity *et al.*, 2013). Trials targeting X-linked adrenoleukodystrophy (ALD) are also relatively advanced, with a Phase II/III trial currently underway (http://clinicaltrials.gov/show/NCT01896102 accessed 08.10.13). ALD is a fatal demyelinating disease caused by mutations in the *ABCD1* (adenosine triphosphate (ATP)-binding cassette, sub-family D, member 1) gene, for which allogeneic haematopoietic cell transplantation from a matched donor is presently the only effective treatment (Cartier *et al.*, 2009). In the recently opened (August 2013) Phase II/III trial, boys with childhood cerebral ALD will receive autologous $CD4^+$ haematopoietic stem cells that have been modified *ex vivo* by a HIV-1-derived vector encoding the *ABCD1* gene (Cartier *et al.* (2009); http://clinicaltrials.gov/show/NCT01896102 accessed 08.10.13). As for gene therapies overall (Section 1.2.3), while lentiviral vectors make good progress in the clinic, developing scalable, GMP-compliant manufacturing processes remains a major challenge (Ansorge *et al.*, 2010; Schweizer and Merten, 2010; Segura *et al.*, 2013). This topic will be covered in depth in Section 1.4; first, however, an introduction to the lentiviral vector utilised as a model system throughout this research is required.

Table 1.5 Indications addressed in lentiviral vector-based clinical trials performed up to January 2013. The number of clinical trials in each Phase is also given. Information was derived from Segura *et al.* (2013); note that this source listed details of 52 trials only.

Indications	Phases					
	I	I/II	II	II/III	III	All
Monogenic diseases						**22**
Adrenoleukodystrophy (ALD)		1		1	1	
Thalassemia	3					
Wiskott-Aldrich syndrome (WAS)	2	1				
ADA¯ SCID		2				
Fanconi anaemia	2					
SCID-X1	2					
Haemophilia A	1					
Metachromatic leukodystrophy		1				
Mucopolysaccharidosis Type VII (MPS VII)	1					
Netherton syndrome	1					
Sickle cell anaemia	1					
Sickle cell anaemia, thalassemia		1				
X-linked chronic granulomatous disease (X-CGD)		1				
Cancer diseases						**15**
Leukaemia / lymphoma	4	2				
Melanoma	3					
Ovarian cancer	2					
Advanced myeloma	1					
Glioma	1					
Paediatric patients with high risk brain tumours	1					
Synovial sarcoma	1					
Infectious diseases						**9**
HIV infection	4	4	1			
Ocular diseases						**4**
Stargardt macular degeneration (SMD)		2				
Age-related macular degeneration (AMD)	1					
Usher Syndrome		1				
Neurological diseases						**2**
Parkinson's disease		2				

1.3.3 ProSavin®: a lentiviral vector for treatment of Parkinson's disease

The research presented in this thesis was predominantly conducted using ProSavin® as a model system. ProSavin® is an EIAV-derived lentiviral vector developed for the treatment of Parkinson's disease, which encodes the enzymes necessary for dopamine synthesis (Mitrophanous *et al.*, 1999; Azzouz *et al.*, 2002; Jarraya *et al.*, 2009; Stewart *et al.*, 2009; Stewart *et al.*, 2011; Palfi *et al.*, 2014). Most EIAV sequences have been removed, leaving a minimal vector system as illustrated in Figure 1.3.

Wild-type EIAV has the simplest genome of any lentivirus (8.2 kb in length), encoding only the genes *gag*, *pol*, *env*, *tat*, *rev* and *S2* (Leroux *et al.*, 2004). *S2* encodes a protein unique to EIAV (Rohll *et al.*, 2002), not required for *in vitro* replication (Li *et al.*, 1998), but of importance for *in*

vivo replication and pathogenicity (Li *et al.*, 2000). It has been suggested that S2 may function in the cytoplasm by organising Gag proteins during particle assembly, and is unlikely to play a role in the early stages of the life cycle (Yoon *et al.*, 2000). Another important difference to HIV-1, is that EIAV, along with all other non-primate lentiviruses, expresses a dUTPase (DU) from a domain within the *pol* gene (Elder *et al.*, 1992), which serves to minimise the misincorporation of uracil into DNA (Chen *et al.*, 2002). It has been demonstrated that S2 and DU are dispensable when creating EIAV-based vectors (Mitrophanous *et al.*, 1999).

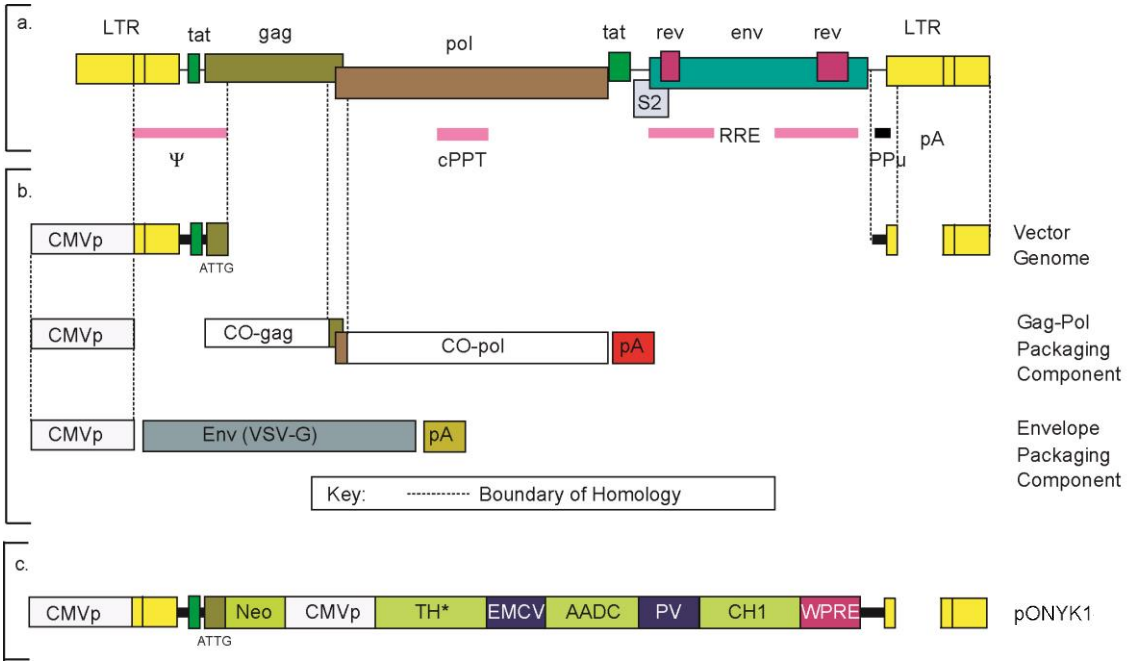


Figure 1.3 Schematic representation of the genetic organisation of (a) wild-type equine infectious anaemia virus (EIAV), (b) lentiviral vector platform, and (c) the ProSavin® genome construct (pONYK1). The following terms have been abbreviated: long terminal repeat (LTR), packaging signal (Ψ), central polypurine tract (cPPT), Rev response element (RRE), polypurine tract (PPu), polyadenylation signal (pA), Cytomegalovirus promoter (CMVp), codon optimised (CO), vesicular stomatitis virus glycoprotein (VSV-G), neomycin resistance gene (neo), tyrosine hydroxylase type 2 gene (TH*), aromatic amino acid decarboxylase gene (AADC), GTP-cyclohydrolase 1 gene (CH1), internal ribosome entry site (IRES) from encephalomyocarditis (EMCV), IRES from polio virus (PV), woodchuck hepatitis virus post transcriptional regulatory element (WPRE). Image kindly provided by Oxford BioMedica. Permission to reproduce this image has been granted by Oxford BioMedica.

The EIAV lentiviral vector system developed by Oxford BioMedica and illustrated in Figure 1.3, comprises three components: the packaging construct, envelope construct, and vector genome construct. In order from 5' to 3' the packaging construct contains a cytomegalovirus promoter (CMVp), a codon-optimised open reading frame (ORF) for Gag-Pol, linked by a non-codon-optimised ribosome shift frame (essential for maintaining the correct balance of Gag and Gag-Pol proteins), and a heterologous polyadenylation (pA) signal. pA signals promote polyadenylation (important for the nuclear export and stability of eukaryotic mRNAs) and terminate transcription of genomic lentiviral RNA (Tolmachov *et al.*, 2011). The envelope component contains (from 5' to 3') a CMVp, an ORF for VSV-G, and a heterologous pA signal. The ProSavin® vector genome construct pONYK1 contains just 861 nucleotides of EIAV sequence and does not express any functional viral proteins. This construct comprises (from 5' to 3') a CMVp-R-U5 region, a packaging signal (Ψ), a neomycin resistance gene (*neo*) ORF (which, by inclusion, abolishes the requirement for *rev* (Miskin *et al.*, 2008)), an internal CMVp, a codon-optimised ORF for truncated tyrosine hydroxylase (TH), an internal ribosome entry site (IRES), a codon-optimised ORF for aromatic amino acid decarboxylase (AADC), another IRES, a codon-optimised ORF for GTP-cyclohydrolase 1 (CH1), a WPRE, and a SIN LTR. TH, AADC and CH1 are the three critical enzymes required for dopamine synthesis (Azzouz *et al.*, 2002). By incorporating IRES units within the transgene cassette, translation of mRNA may be initiated from more than one site (Hellen and Sarnow, 2001).

As ProSavin® is the lentiviral vector predominantly employed in the studies presented in this thesis, a brief introduction to Parkinson's disease and current treatment options is included; however, it is important to note that several products have been generated using the EIAV lentiviral vector platform (Table 1.6). ProSavin® was chosen as a model system as it is the most clinically advanced (Phase I/II trials have been completed, see Palfi *et al.* (2014)), and it is anticipated that large amounts of material will be required for Phase III trials and commercial manufacture, thus the speedy development of a scalable bioprocess is highly desirable (Stewart *et al.*, 2011). Parkinson's disease is the second most prevalent progressive neurodegenerative disorder after Alzheimer's disease (Nanou and Azzouz, 2009). The average age at onset is 60 years (Ishihara *et al.*, 2007) and in age groups greater than 60 years prevalence was reported to be 1.28 - 1.5 % in European countries (von Campenhausen *et al.*, 2005). For Western

Europe's five most and the world's ten most populous nations Dorsey *et al.* (2007) estimated the total number of individuals greater than 50 years with Parkinson's disease to be 4.1 - 4.6 million in 2005 and forecasted that this figure would double to 8.7 - 9.3 million by 2030. The precise aetiology of Parkinson's disease is unknown (Olanow and Tatton, 1999) and a consequent lack of reliable predictive risk markers has hindered the development of effective preventative treatments (Poewe, 2009). However, the pathogenesis of Parkinson's disease has been well described. Parkinson's disease is chiefly characterised by a loss of dopaminergic neurons in the *substantia nigra pars compacta* region of the brain, which leads to a depletion of dopamine in the striatum (Nanou and Azzouz, 2009). Archetypal clinical symptoms include rigidity, resting tremor, bradykinesia and postural instability; although a multitude of motor and non-motor symptoms can accompany the disease (Jankovic, 2008).

Table 1.6 Pipeline of products developed by Oxford BioMedica that are based on the equine infectious anaemia virus (EIAV) lentiviral vector platform. Information was derived from Oxford BioMedica (2013).

Indication	Product	Partner / funding	Development stage
Neurological			
Parkinson's disease	ProSavin®	-	Phase I/II trial completed
Motor neuron disease	MoNuDin®	UK Motor Neurone Disease Association	Research
Ocular			
Stargardt disease	StarGen™	Sanofi	Phase I/IIa trial ongoing
Usher syndrome type 1B	UshStat®	Sanofi	Phase I/IIa trial ongoing
Corneal graft rejection	EncorStat®	Sanofi	Phase I/II trial preparation
"Wet" age-related macular degeneration	RetinoStat®	Sanofi	Phase I trial ongoing
Chronic glaucoma	Glaucoma-GT	Mayo Clinic, USA	Pre-clinical

For over 40 years, levodopa has been the gold standard treatment for controlling the symptoms of Parkinson's disease (Poewe, 2009). Levodopa is the amino acid precursor to dopamine which, unlike dopamine, can cross the blood-brain barrier (Pezzoli and Zini, 2010). Chronic use of levodopa is associated with disabling motor complications, however, which emerge after approximately five years of therapy (Yokochi, 2009). These complications arise due to the discontinuous delivery of levodopa to the brain (a function of the oral dosage regimen), which

leads to the pulsatile stimulation of dopamine receptors (Olanow *et al.*, 2006). Attempts to define alternative delivery routes for levodopa have produced limited success, and a decrease in the efficacy of levodopa therapy over time remains inevitable due to a continuing degeneration of dopaminergic neurons with disease progression, thus cell replacement therapies and gene therapies are being evaluated as alternatives (Goole and Amighi, 2009).

At present, cell replacement approaches appear to have been delayed, as early clinical studies failed to demonstrate efficacy and, worryingly, caused graft-induced dyskinesia in some patients (Lindvall, 2013). Gene therapy has shown more promise, with four approaches having recently undergone clinical evaluation. Three of these employed adeno-associated serotype 2 virus vectors to deliver genes encoding either (i) glutamic acid dehydroxylase (GAD), which catalyses production of the neurotransmitter γ-aminobutyric acid (GABA) (Kaplitt *et al.*, 2007; LeWitt *et al.*, 2011), (ii) the neurotropic factor neurturin (Marks *et al.*, 2010), or (iii) the AADC enzyme (evaluated by two groups) (Muramatsu *et al.*, 2010; Valles *et al.*, 2010). The fourth approach - based on the lentiviral vector ProSavin® - is the only strategy to deliver all three enzymes required for dopamine synthesis (AADC, CH1, TH) to the striatum (Jarraya *et al.*, 2009). Promising data pertaining to the safety and efficacy of ProSavin® was generated in a Phase I/II study (Palfi *et al.*, 2014) and Oxford BioMedica are presently evaluating a more potent vector construct to maximise the likelihood of success in future Phase II trials (Oxford BioMedica, 2012). A further challenge is the development of a bioprocess that is capable of generating sufficient material to satisfy future Phase III and commercial requirements (Stewart *et al.*, 2011), and methods for the manufacture of lentiviral vectors are discussed in depth below.

1.4 Manufacturing lentiviral vectors

The establishment of large-scale clinical GMP-compliant bioprocesses for lentiviral vector-based gene therapies is a pressing challenge (Ansorge *et al.*, 2010; Schweizer and Merten, 2010; Segura *et al.*, 2013). This section considers current and upcoming lentiviral vector manufacturing approaches, and their suitability for commercial application. Pertinent issues were highlighted in Section 1.2.3 for gene therapies in general and, as these issues are wholly relevant for lentiviral vector-derived therapies, they shall be reiterated here in brief: (i) the use of adherent cell expression systems is not scalable, (ii) elimination of contaminants arising from

the upstream process, such as medium components (e.g. serum), those derived from transient transfections (including plasmid DNA), and those derived from host cells is problematic, and impossible if they have been incorporated into viral particles, (iii) removal of non-functional vector is difficult as it often co-purifies with functional vector, and (iv) concentration to the degree required for therapeutic purposes without co-concentration of impurities is challenging. It is with these concerns in mind that recent progress in the area of lentiviral vector manufacturing is presented. This section covers cell expression systems, culture technologies, downstream processing methodologies and assays for evaluation of the quantity and quality of lentiviral vector yields. The section concludes by considering the development of future manufacturing approaches for ProSavin®, in terms of progress made and challenges remaining.

1.4.1 Cell expression systems

For the large-scale manufacture of lentiviral vectors, adherently cultured HEK293 cells or their derivatives are most commonly employed (Ansorge *et al.*, 2010; Schweizer and Merten, 2010; Segura *et al.*, 2013). Exposure of human embryonic kidney cells to sheared Adenovirus Type 5 fragments yielded the immortalised HEK293 cell line (Graham *et al.*, 1977), which contains the adenoviral transcription units early region (E)1a and E1b (Louis *et al.*, 1997). HEK293 cells are preferentially employed for lentiviral production as they are of human derivation, have a proven safety record for production of retroviral vectors (Ansorge *et al.*, 2010), and are easy to transfect (Segura *et al.*, 2007). As multi-plasmid transient transfection is the method most commonly employed for generating research and clinical-grade lentiviral vectors, HEK293T is the most commonly used cell derivative (Cockrell and Kafri, 2007; Schweizer and Merten, 2010; Gama-Norton *et al.*, 2011). HEK293T cells express the Simian vacuolating virus 40 (SV40) large T antigen, which promotes the replication of plasmids carrying the SV40 origin of replication (DuBridge *et al.*, 1987). Use of HEK293T as compared to HEK293 has been reported to improve titres by around 4- to 10-fold (Merten *et al.*, 2011; Ausubel *et al.*, 2012).

HIV-1 based lentiviral vectors have several proteins reported to be cytotoxic or cytostatic, including: protease (Kaplan and Swanstrom, 1991), Rev (Miyazaki *et al.*, 1995), Tat (Li *et al.*, 1995), Vpr (Planelles *et al.*, 1995; Rogel *et al.*, 1995) and Env (Sodroski *et al.*, 1986). Although Tat, Vpr and Env are not expressed in third generation lentiviral vectors (Kim *et al.*, 1998), the

problem is not necessarily reduced as, for example, the VSV-G envelope commonly used as a replacement for viral Env is also highly cytotoxic (Burns *et al.*, 1993; Yee *et al.*, 1994; Yang *et al.*, 1995; Ory *et al.*, 1996). As constitutive expression of lentiviral vectors is thus not possible, transient transfection of cells with plasmid DNA has been the method most utilised to generate lentiviral vectors to date (Cockrell and Kafri, 2007; Schweizer and Merten, 2010; Gama-Norton *et al.*, 2011; Segura *et al.*, 2013). Using this technique, transfected DNA remains in the cell nucleus in an episomal state until cell death occurs, presumably due to the accumulation of cytotoxic vector proteins (Al-Dosari and Gao, 2009). GMP clinical productions have been performed using two (Slepushkin *et al.*, 2003), three (Stewart *et al.*, 2009), four (Merten *et al.*, 2011) or five plasmids (Negré *et al.*, 2008); multiple plasmids are used to minimise the likelihood of recombination leading to RCL, as noted earlier (Section 1.3.2). Published protocols for the production of GMP clinical grade material are summarised in Table 1.7. As naked DNA molecules cannot efficiently traverse cell membranes due to their large size and hydrophilic nature, delivery of DNA into the cytoplasm is accomplished using either chemical compounds or physical force (Al-Dosari and Gao, 2009). Once in the cytoplasm, nuclear entry of DNA is best achieved in dividing cells, where the nuclear envelope disassembles during mitosis, removing the barrier (Dean *et al.*, 2005). To gain cellular entry, co-precipitation of DNA molecules with calcium phosphate is the technique most often employed for clinical GMP lentiviral vector production (Slepushkin *et al.*, 2003; Negré *et al.*, 2008; Merten *et al.*, 2011; Ausubel *et al.*, 2012). Interestingly, ProSavin® for Phase I/II trials was generated using a cationic liposome-mediated transfection (lipofection) method (Stewart *et al.*, 2011), although the cost of the commercial reagent is thought to preclude use of this method for large-scale productions (Segura *et al.*, 2013). Partly due to this concern stable producer cell lines are in development for the manufacture of ProSavin® for Phase III trials and beyond (Stewart *et al.*, 2009; 2011). Alternative transfection methods that have shown recent potential, but to the best of my knowledge have not yet been applied in clinical setting, include the use of polyethylenimine (PEI) (Kuroda *et al.*, 2009; Toledo *et al.*, 2009; Segura *et al.*, 2010) and electroporation (Witting *et al.*, 2012).

Table 1.7 Published upstream processing protocols employed for large-scale production of Good Manufacturing Practice (GMP)-compliant clinical-grade lentiviral vectors. VIRxSYS's (Gaithersburg, MD, USA) protocol relates to the first clinical lentiviral vector, VRX496™, used for treatment of HIV; Oxford BioMedica's protocol relates to ProSavin® used for treatment Parkinson's disease; Généthon's (Evry, France) protocol relates to a lentiviral vector used for treatment of Wiskott-Aldrich Syndrome (WAS); Beckman Research Institute's (Duarte, CA, USA) protocol relates to a lentiviral vector used for treatment of HIV. TU is an abbreviation for 'transducing units' and IG is an abbreviation for 'infectious genomes'.

Institute / company	VIRxSYS	Oxford BioMedica	Généthon	Beckman Research Institute
Reference	Slepushkin *et al.* (2003)	Schweizer and Merten (2010); Stewart *et al.* (2011)	Merten *et al.* (2011)	Ausubel *et al.* (2012)
Lentiviral vector	HIV-1	EIAV	HIV-1	HIV-1
Cell line	HEK293	HEK293T	HEK293T	HEK293T
Number of plasmids	2	3	4	4
Transfection reagent	Calcium phosphate	Lipofectamine™ 2000 CD	Calcium phosphate	Calcium phosphate
Vessel	10-layer Cell Factory™	10-layer Cell Factory™	10-layer Cell Factory™	10-layer Cell Factory™
Production scale (L)	36-52	72 (24 per campaign)	52	20-120
Crude titre	1.1-3.2 x 10^7 TU mL^{-1}	0.2-2.0 x 10^6 TU mL^{-1}	3.1-5.0 x 10^7 IG mL^{-1}	~1 x 10^6 TU mL^{-1}

Transient transfection methods offer flexibility and are fast to develop, thus are suited to producing lentiviral vectors for early Phase clinical trials (Ansorge *et al.*, 2010; Rodrigues *et al.*, 2011; Segura *et al.*, 2013). However, for generating licensed product, use of this technique is unfeasible for the following reasons: (i) it is costly and technically challenging to scale-up (ii) batch-to-batch variability is common due to variability in plasmid uptake and expression efficiencies (iii) a transfection step complicates the upstream process and the elimination of plasmid DNA downstream is difficult, (iv) the presence of large quantities of transfected plasmid DNA may increase the risk of a recombination event leading to formation of a RCL (Cockrell and Kafri, 2007; Stewart *et al.*, 2009; Ansorge *et al.*, 2010; Schweizer and Merten, 2010; Rodrigues *et al.*, 2011; Segura *et al.*, 2013). As a result, stable packaging and producer cell lines are in development (Table 1.8) which, although initially time consuming to create (Cockrell and Kafri, 2007; Ansorge *et al.*, 2010; Rodrigues *et al.*, 2011), should ultimately address these concerns. Packaging cell lines express all lentiviral vector components apart from the genome

construct, thus still require one-plasmid transient transfection; producer cell lines, however, express all necessary lentiviral vector components (Ansorge *et al.*, 2010). Controlled gene expression is essential for overcoming the cytotoxic/cytostatic effects of certain lentiviral vector components, and for this purpose a tetracycline(tet)-OFF regulatory system has been most commonly employed (Table 1.8). However, as total removal of doxycycline (dox; an analogue of tet used to maintain cells in the OFF state) is only practical with adherent cells, these systems are not considered amenable to large-scale production (Ansorge *et al.*, 2010). As a consequence packaging and producer cells incorporating a tet-ON regulatory system have recently been developed (Broussau *et al.*, 2008; Stewart *et al.*, 2009). Encouragingly, titres from these systems were in-line with those attained using current clinical GMP transient transfection protocols (Table 1.7), and the producer clones (encoding green fluorescent protein [GFP] or ProSavin®, respectively) were stable during extended cultures (≥ 83 days) in the absence of antibiotic selection (Broussau *et al.*, 2008; Stewart *et al.*, 2011). The cell lines developed by Broussau *et al.* (2008) were grown in serum-free suspension conditions, thus this study represents the most promising advance towards a scalable upstream process for lentiviral vectors to date. Large-scale culture systems that have been utilised for the production of clinical-grade lentiviral vectors, and studies evaluating alternative vessels for future applications are outlined below.

Table 1.8 Comparison of packaging and producer cell lines for lentiviral vector production. The maximum titre from a single harvest is reported. ^{PACK} or ^{PROD} indicates that a given value refers to a packaging or producer cell line, respectively. CFU, IU and TU are abbreviations of 'colony forming units', 'infectious units', and 'transducing units', respectively.

Parental cell line	Regulatory system	Vector	Culture mode	Culture scale	Titre	Reference
HeLa	Tet-OFF	HIV-1	Adherent	6 cm dish (21 cm^2)	7 x 10^3 CFU $mL^{-1\ PACK}$	Yu *et al.* (1996)
HeLa	Tet-OFF	HIV-1	Adherent	10 cm dish (57 cm^2)	3.5 x 10^4 IU $mL^{-1\ PACK}$	Kaul *et al.* (1998)
HEK293	Tet-OFF	HIV-1	Adherent	10 cm dish (57 cm^2)	3 x 10^6 IU $mL^{-1\ PROD}$	Kafri *et al.* (1999); Xu *et al.* (2001)
HEK293	Tet-OFF	HIV-1	Adherent	10 cm dish (57 cm^2)	5 x 10^6 TU $mL^{-1\ PROD}$	Klages *et al.* (2000)
HEK293	Tet-OFF	HIV-1	Adherent	10 cm dish (57 cm^2)	5.1 x 10^5 TU $mL^{-1\ PACK}$ 6.6 x 10^6 TU $mL^{-1\ PROD}$	Farson *et al.* (2001)
HEK293T	Ecdysone-ON	HIV-1	Adherent	10 cm dish (57 cm^2)	1.2 x 10^5 IU $mL^{-1\ PROD}$	Pacchia *et al.* (2001)
HEK293	Ecdysone-ON	HIV-1	Adherent	No information	3 x 10^5 IU $mL^{-1\ PACK}$	Sparacio *et al.* (2001)
HEK293T	None	HIV-1	Adherent	No information	1.4 x 10^7 IU $mL^{-1\ PACK}$	Ikeda *et al.* (2003)
HEK293	Ecdysone-ON	SIV	Adherent	182 cm^2 dish	2.6 x 10^5 TU $mL^{-1\ PACK}$ 2.4 x 10^5 TU $mL^{-1\ PROD}$	Kuate *et al.* (2002)
HEK293	Tet-OFF	HIV-1	Adherent	Cell factory (6320 cm^2)	3.5 x 10^7 TU $mL^{-1\ PACK}$	Ni *et al.* (2005)
HEK293	Tet-OFF	HIV-1	Adherent	10 cm dish (57 cm^2)	2.4 x 10^6 TU $mL^{-1\ PACK}$ 1 x 10^7 TU $mL^{-1\ PROD}$	Cockrell *et al.* (2006)
HEK293	Tet-ON + cumate	HIV-1	Suspension	Stirred bioreactor (2.8 L) ^{PACK} Shake flask (25 mL) ^{PROD}	2 x 10^6 TU $mL^{-1\ PACK}$ 3.4 x 10^7 TU $mL^{-1\ PROD}$	Broussau *et al.* (2008)
HEK293T	Tet-ON	EIAV	Adherent	10 cm dish (57 cm^2)	1.6 x 10^6 TU $mL^{-1\ PACK}$ 4.4 x 10^5 TU $mL^{-1\ PROD}$	Stewart *et al.* (2009); Stewart *et al.* (2011)
HEK293T	Tet-OFF	HIV-1	Adherent	10 cm dish (57 cm^2) ^{PACK} Wave bioreactor (25 L) ^{PROD}	5 x 10^7 TU $mL^{-1\ PACK}$ 2 x 10^7 TU $mL^{-1\ PROD}$	Throm *et al.* (2009); Greene *et al.* (2012)
HEK293T	Tet-OFF	HIV-1	Adherent	10 cm dish (57 cm^2)	3 x 10^7 TU $mL^{-1\ PROD}$	Lee *et al.* (2012)
HEK293T	None	HIV-1	Adherent	10 cm dish (57 cm^2)	3 x 10^5 TU $mL^{-1\ PACK}$ 1.2 x 10^6 TU $mL^{-1\ PROD}$	Stornaiuolo *et al.* (2013)

1.4.2 Large-scale cell culture systems

GMP-compliant lentiviral vector productions are currently based on transient transfection of adherent HEK293-derived cell lines (Section 1.4.1), and to generate sufficient material for early Phase clinical trials the Nunc™ Cell Factory™ (Thermo Fisher Scientific, Wilmington, DE, USA) is the only system reported to have been employed to date (Slepushkin *et al.*, 2003; Schweizer and Merten, 2010; Merten *et al.*, 2011; Stewart *et al.*, 2011; Ausubel *et al.*, 2012). Cell Factory™ systems are simply multi-layered tissue culture trays, each tray has a culture area of 632 cm^2, and usually the trays are utilised as 10-layer stacks (Table 1.7). Alternative large-scale culture vessels that have been evaluated for lentiviral vector production, but have not yet been utilised to generate clinical-grade material are given in Table 1.9.

Table 1.9 Comparison of large-scale culture methods that have been evaluated for lentiviral vector production. Alternatives to the Cell Factory™ system conventionally used for clinical-grade manufacturing are listed. The maximum titre from a single harvest is reported. TU, IU and IVP are abbreviations of 'transducing units', 'infectious units' and 'infectious viral particles', respectively.

Vessel	Scale	Growth mode	Process	Titre	Reference
HYPER*Flask*™	1720 cm^2 (single flask)	Adherent monolayer	3 plasmid transient transfection using calcium phosphate	2.3 x 10^8 TU mL^{-1}	Kutner *et al.* (2009)
HYPER*Flask*™	1720 cm^2 (single flask)	Adherent monolayer	3 plasmid transient transfection using polyamine	1.3 x 10^7 TU mL^{-1}	Cooper *et al.* (2011)
WAVE bioreactor	25 L	Adherent attached to microcarriers	Tet-OFF producer cell line	2 x 10^7 TU mL^{-1}	Throm *et al.* (2009); Greene *et al.* (2012)
WAVE bioreactor	2.3 L	Serum-free suspension	4 plasmid transient transfection using flow electroporation	1.3 x 10^7 IU mL^{-1}	Witting *et al.* (2012)
Stirred tank bioreactor	3 L	Serum-free suspension	4 plasmid transient transfection using PEI	1.1 x 10^6 IVP mL^{-1}	Segura *et al.* (2007)
Stirred tank bioreactor	2.8 L	Serum-free suspension	Tet-ON (+ cumate) packaging cell line, single plasmid transient transfection using PEI	2 x 10^6 TU mL^{-1}	Broussau *et al.* (2008)
Stirred tank bioreactor	2.7 L	Serum-free suspension supplemented with bovine serum albumin	4 plasmid transient transfection using PEI	8 x 10^7 TU mL^{-1}	Ansorge *et al.* (2009)

Use of the Corning® HYPER*Flask*™ cell culture vessel (Sigma-Aldrich, Poole, UK) for lentiviral vector production has been reported (Kutner *et al.*, 2009; Cooper *et al.*, 2011). HYPER*Flask*™ vessels comprise ten growth areas (totalling 1720 cm^2) that are each interconnected by a gas permeable membrane, and provide the benefit of a small footprint (similar to a conventional 175 cm^2 tissue culture flask). Kutner *et al.* (2009) reported approximately 10-fold higher productivity when generating lentiviral vectors by calcium phosphate transient transfection in a HYPER*Flask*™ as compared to a 150 cm^2 dish (which is analogous to a Cell Factory™ in terms of the culture environment) that the authors hypothesised could be due to superior gas exchange. Further work is necessary to confirm whether this improvement holds true for other lentiviral vector expression systems. However, despite potential advantages over the Cell Factory™ system in terms of equipment footprint and attainable titres, neither vessel is suitable for commercial scale manufacture, as production capacity can only be increased by linear scale-out, i.e. by multiplying the number of production vessels (Schweizer and Merten, 2010).

Growth of adherent cells on microcarrier disks, as opposed as to monolayers, overcomes the issue of linear scale-out. It was recently reported that stable producer cells that were adhered to Fibra-Cel® microcarrier disks (New Brunswick, Enfield, CT, USA) could be cultured at 25 L scale and generated lentiviral vectors at clinically relevant titres (Throm *et al.*, 2009; Greene *et al.*, 2012). A drawback of the reported process, however, was the use of a tet-OFF regulatory system, as multiple wash steps and a full medium exchange was thus required for cell induction (Throm *et al.*, 2009; Greene *et al.*, 2012). These studies employed a WAVE bioreactor system (GE Healthcare, Buckinghamshire, UK), in which cells are cultured within a disposable bag chamber and are mechanically rocked to facilitate fluid mixing and oxygen transfer. It is likely this form of bioreactor was chosen as bubble-free aeration and minimal mechanical stress is required for the efficient growth of microcarrier cultures (Ansorge *et al.*, 2010). An added benefit of the WAVE system is that, along with the Cell Factory™ and HYPER*Flask*™ vessels, it is single-use. For the production of lentiviral vectors, disposable technologies are preferable to re-usable systems as lentiviral vectors cannot be subjected to traditional virus inactivation or removal steps (such as those used for recombinant proteins), thus contamination by adventitious agents must be avoided during their manufacture (Segura *et al.*, 2013). The pre-sterility of the cultivation vessel also affords cost and time savings and allows flexible

application of the bioreactor base unit with a minimal chance of cross-contamination (Eibl *et al.*, 2010b). A potential drawback with both single-use technologies and microcarrier disks is the risk of generating extractables and leachables. The use of WAVE bioreactors is more commonplace, and the 'Cellbag' cultivation chamber meets US Pharmacopeia Class VI testing for leachables (Mikola *et al.*, 2007), however the use of microcarriers can generate fine debris and contaminants that are difficult to remove downstream, and are of particular concern when seeking to develop a GMP-compliant process.

Despite the recent advances made using microcarriers (Throm *et al.*, 2009; Greene *et al.*, 2012), scale-up is most cost-effectively and easily achieved using suspension-adapted cell lines (Peshwa *et al.*, 1993; Ansorge *et al.*, 2010), thus this remains the best option for future industrial productions of lentiviral vectors. However, HEK293 cells and their derivatives display a high propensity for aggregation when cultured in suspension (Wurm and Bernard, 1999; Liu *et al.*, 2006; Zhao *et al.*, 2007), an undesirable trait as cells in the centre of clumps gradually become non-viable, possibly due to diffusional limitations (Peshwa *et al.*, 1993), likely limiting the overall productivity of the culture. Cell density, extent of agitation, magnesium and calcium concentrations have all been reported to affect the degree of HEK293 aggregation observed in suspension cultures (Peshwa *et al.*, 1993; Liu *et al.*, 2006; Zhao *et al.*, 2007; Liu *et al.*, 2009). Lowering the calcium content of culture media has been the main strategy employed to minimise the formation of HEK293 aggregates, and the creation of specialist suspension media, such as the low-calcium serum-free medium (LC SFM) developed by Côté *et al.* (1998), has enabled suspension-based lentiviral vector production strategies to be developed (Table 1.9).

Witting *et al.* (2012) presented a large-scale process for lentiviral vector production that was based on the culture of suspension-adapted HEK293-derived cells in a WAVE bioreactor system. However, the complexity of the protocol would likely preclude its industrial application. Although cells were initially expanded in the WAVE bioreactor (to 5 L), they were then removed, pelleted, washed, re-suspended in 10 - 100 mL and transfected in an electroporation chamber using four plasmids, following which the suspension was transferred into multiple tissue culture flasks and DNase treated, before finally being returned to the WAVE bioreactor (fill volume 2.1 - 2.3 L) for two days, after which lentiviral vector could be harvested (Witting *et al.*, 2012). The number of manipulations involved makes this process vulnerable to contamination and,

although the authors acknowledge that improvements to the liquid handling steps are required (Witting *et al.*, 2012), overall the use transient transfection makes the process unattractive for commercial applications.

Conventional stirred bioreactors have also been explored for the production of lentiviral vectors from suspension cultures (Segura *et al.*, 2007; Broussau *et al.*, 2008; Ansorge *et al.*, 2009). An advantage of stirred bioreactors, as compared to WAVE systems, is that they have long standing use in the biotechnology sector, thus the culture environment has been highly characterised. Segura *et al.* (2007) produced lentiviral vectors from suspension cultures of HEK293-derived cells in a 3 L bioreactor by PEI-mediated transient transfection. Titres were in-line with those attained using standard adherent protocols however, to realise most benefit from the system, three full media exchanges were performed (Segura *et al.*, 2007), complicating the procedure. Again, the use of transient transfection renders this process unrealistic for commercial applications. A significant breakthrough was made by Broussau *et al.* (2008), who generated lentiviral vectors in a 2.8 L stirred bioreactor using a batch suspension culture of HEK293-derived packaging cells. To date, no other study has reported the successful development of a lentiviral vector packaging or producer cell line that is capable of growth in suspension (Table 1.8), likely as changes in cell morphology brought about by suspension adaption can diminish the capacity of these cells to generate high titres (Ansorge *et al.*, 2010). The success achieved by Broussau *et al.* (2008) could be owing to the fact packaging and producer clones were generated from cells that had previously been adapted to suspension, thus subsequent to stable transfection no further adaption was required. Reasonable titres were generated from the packaging cells in the 2.8 L bioreactor (Table 1.9), although these were more than three-fold lower than those attained from 25 mL shake flask cultures, which the authors suggested may have been because transfection efficiency is generally poorer at larger scales (Broussau *et al.*, 2008). It should be noted that producer clones were also developed, which it is conceivable might therefore outperform the packaging cells in the stirred bioreactor, however this was not tested (Broussau *et al.*, 2008). Finally, Ansorge *et al.* (2009) generated lentiviral vectors in a 2.7 L stirred bioreactor by PEI-mediated transfection of suspension-adapted HEK293. This process was thus analogous to that reported earlier by Segura *et al.* (2007), and suffers the same drawback of still relying on transient transfection, limiting its

applicability primarily to early Phase clinical trials. However, an important advance was the development of a perfused system, which is more industrially viable than discontinuous media exchanges, such as were performed by Segura *et al.* (2007). Lentiviral vectors rapidly decay at 37 °C, the temperature at which they are produced (Higashikawa and Chang, 2001; Carmo *et al.*, 2009a; Carmo *et al.*, 2009b), thus perfusion enables their continual removal to low temperature storage (preserving their activity), while also preventing the accumulation of toxic by-products within the cell media and introducing new nutrients to extend the productivity of the culture. Overall, the perfused system generated good titres (Table 1.9) although similar to Broussau *et al.* (2008), the authors found that titres were lower in the bioreactor than those generated using small-scale cultures, which they also suggested was a result of less efficient transfection, but went further to hypothesise that perhaps this was due to increased cell aggregation caused by the acoustic cell filter used (Ansorge *et al.*, 2009). Overall, the potential of stirred bioreactors for lentiviral vector production has been demonstrated and, although non-disposable vessels were used in these particular studies (Segura *et al.*, 2007; Broussau *et al.*, 2008; Ansorge *et al.*, 2009), several disposable stirred bioreactors have been developed (Eibl *et al.*, 2010a) which may prove superior for future industrial applications.

It is clear from the paucity of available data regarding scalable bioprocesses for lentiviral vector production (Table 1.9) that this topic is in its infancy. This is probably because, so far, lentiviral vectors have mostly been employed in early Phase clinical trials (Table 1.5) that require a relatively small volume of material, thus methods based on the transient transfection of adherent monolayers of HEK293-derived cells have proved adequate. However, it is now more than 10 years since the first gene therapy trial using lentiviral vectors commenced, and lentiviral vectors have since made good progress in the clinic (Section 1.3.2), as such the apparent lack of scalable, GMP-compliant manufacturing processes is of mounting concern (Ansorge *et al.*, 2010; Schweizer and Merten, 2010; Segura *et al.*, 2013). An ideal upstream process would be based on serum-free suspension cultures of ON-inducible stable producer cells, and would generate high-quality (i.e. containing a low proportion of defective particles) high-titre lentiviral vectors within a disposable closed system. To date, no single published process has successfully combined all of these elements, although several studies have reported progress in one or more of these areas (Segura *et al.*, 2007; Broussau *et al.*, 2008; Ansorge *et al.*, 2009;

Stewart *et al.*, 2009; Throm *et al.*, 2009; Stewart *et al.*, 2011; Greene *et al.*, 2012; Witting *et al.*, 2012). The development of industrially viable upstream processes for lentiviral vectors is thus an area warranting significant attention.

1.4.3 Scalable downstream processing methodologies

Although lentiviral vector downstream processing strategies are not explored in this research, in order to design efficient upstream processes that do not overburden subsequent purification and concentration stages an understanding of this topic is critical, thus a brief overview of current practices is here presented. The primary goal of downstream processing specific to gene therapy is to remove impurities while retaining vector activity (Segura *et al.*, 2013). Impurities may be product-related, e.g. non-functional vector particles; or process-related, such as those derived from the culture medium, production process (e.g. transfection reagents) and host cells, or extractables and leachables derived from the application of single-use technologies during either upstream or downstream processing steps (Segura *et al.*, 2013). Published protocols that have been utilised to prepare material for early Phase clinical trials are presented in Table 1.10. The unit operations employed varied slightly in each instance, as did the order in which they were performed; however the overall approach was not dissimilar.

Table 1.10 Published downstream processing protocols used for the large-scale preparation of GMP-compliant clinical-grade lentiviral vectors. The specific product to which each protocol relates was outlined earlier (Table 1.7). Both original (left column) and improved (right column) downstream approaches reported by VIRxSYS (Slepushkin *et al.*, 2003) are given. TU is an abbreviation for 'transducing units' and IG is an abbreviation for 'infectious genomes'.

Institute / company	VIRxSYS		Oxford BioMedica	Généthon	Beckman Research Institute
Reference	Slepushkin *et al.* (2003); Lu *et al.* (2004); Schweizer and Merten (2010)		Schweizer and Merten (2010); Stewart *et al.* (2011)	Merten *et al.* (2011)	Ausubel *et al.* (2012)
Process steps	***Clarification*** (filtration using decreasing pore size to 0.22 µm)	***Clarification*** (filtration using decreasing pore size to 0.22 µm)	***Clarification*** (1.2 µm to 0.45 µm filtration)	***Clarification*** (0.8 µm to 0.45 µm filtration)	***Clarification*** (0.45 µm or 0.5 µm filtration)
	Concentration (ultrafiltration) **+** ***buffer exchange*** (diafiltration)	***Purification*** (anion exchange chromatography)	***Nucleic acid digestion*** (Benzonase®)	***Nucleic acid digestion*** (Benzonase®)	***Nucleic acid digestion*** (Benzonase®)
	Nucleic acid digestion (Benzonase®)	***Concentration*** (ultrafiltration) **+** ***buffer exchange*** (diafiltration)	***Purification*** (anion exchange chromatography)	***Purification*** (anion exchange chromatography)	***Concentration*** (ultrafiltration) **+** ***buffer exchange*** (diafiltration)
	Polishing (size exclusion chromatography)	***Nucleic acid digestion*** (Benzonase®)	***Concentration*** (ultrafiltration) **+** ***buffer exchange*** (diafiltration)	***Concentration*** (ultrafiltration)	***Concentration*** (centrifugation)
	Sterilisation (0.22 µm filtration)	***Buffer exchange*** (diafiltration)	***Sterilisation*** (0.2 µm filtration)	***Polishing*** (size exclusion chromatography) **+** ***buffer exchange***	
		Sterilisation (0.22 µm filtration)	***Concentration*** (ultrafiltration)	***Sterilisation*** (0.22 µm filtration)	
Concentration factor (x)	30-40	20	2000	200	209-357
Recovery (%)	30	30	30-40	13	31
Final titre	3.3×10^8 TU mL^{-1}	2.2×10^8 TU mL^{-1}	$0.1\text{-}2.0 \times 10^9$ TU mL^{-1}	$\geq 1 \times 10^9$ IG mL^{-1}	$0.5\text{-}3.0 \times 10^8$ TU mL^{-1}

Clarification of crude harvests to eliminate cells and cellular debris is achieved at large-scale by microfiltration, where the supernatant is commonly passed through several filters of decreasing pore size to minimise membrane fouling. The smallest pore size typically used at this stage is 0.45 µm (Merten *et al.*, 2011; Stewart *et al.*, 2011; Ausubel *et al.*, 2012; Bandeira *et al.*, 2012; Yang *et al.*, 2012), or 0.2/0.22 µm (Slepushkin *et al.*, 2003; Lu *et al.*, 2004). Material for early Phase clinical trials is subjected to Benzonase® (Sigma-Aldrich) treatment to digest unwanted nucleic acids (Slepushkin *et al.*, 2003; Ansorge *et al.*, 2010; Schweizer and Merten, 2010; Merten *et al.*, 2011; Stewart *et al.*, 2011; Ausubel *et al.*, 2012).

After initial harvest and clarification of the vector using membrane filtration, further purification has been most commonly achieved by anion exchange chromatography, which exploits the negative charge of lentiviral vectors (Slepushkin *et al.*, 2003; Ansorge *et al.*, 2010; Schweizer and Merten, 2010; Lesch *et al.*, 2011; Merten *et al.*, 2011; Stewart *et al.*, 2011; Zimmermann *et al.*, 2011), while their large size has also supported the use size exclusion chromatography as a polishing step (Slepushkin *et al.*, 2003; Merten *et al.*, 2011). For the preparation of early clinical material, purification by anion exchange chromatography has generally preceded vector concentration (Table 1.10), perhaps so that co-concentration of impurities may be minimised, however when moving to commercial scale the practicability and cost of purifying such large feed volumes requires consideration. To concentrate lentiviral vectors, centrifugation has been used to prepare early clinical material (Ausubel *et al.*, 2012), although this method is generally not considered scalable (Segura *et al.*, 2006; Ansorge *et al.*, 2010; Segura *et al.*, 2013). At large-scale, ultrafiltration is more commonly used, with tangential flow filtration being the preferred method (Slepushkin *et al.*, 2003; Geraerts *et al.*, 2005; Schweizer and Merten, 2010; Cooper *et al.*, 2011; Merten *et al.*, 2011; Stewart *et al.*, 2011). Ultrafiltration may be combined with diafiltration for buffer exchange (Slepushkin *et al.*, 2003; Lu *et al.*, 2004; Cooper *et al.*, 2011; Stewart *et al.*, 2011; Ausubel *et al.*, 2012). Finally, downstream processing of clinical material usually concludes with sterile filtration, using a pore size of 0.2 or 0.22 µm (Slepushkin *et al.*, 2003; Merten *et al.*, 2011; Stewart *et al.*, 2011), although one group has reported omitting this step (Ausubel *et al.*, 2012), presumably to mitigate losses of functional vector. One protocol included an ultrafiltration step after sterile filtration, for the further concentration of lentiviral vectors (Stewart *et al.*, 2011).

Little data has been published regarding the final formulation of lentiviral vectors. For clinical material, this has been described in two instances, in one case a protein-containing medium required for *ex vivo* delivery of the lentiviral vector was used (Merten *et al.*, 2011) while in the other phosphate buffered saline (PBS) supplemented with lactose was utilised (Ausubel *et al.*, 2012). Lentiviral vectors are usually stored at -80 °C to prevent thermal inactivation (Schweizer and Merten, 2010; Segura *et al.*, 2013). Following downstream processing of clinical-grade material, recoveries were typically only 13 - 40 %, although 20 to 2000-fold concentration was achieved and final yields of at least 1×10^8 TU mL^{-1} attained (Table 1.10).

1.4.4 Assays to quantify lentiviral vectors

At various stages of the manufacturing process, it is desirable to evaluate the quantity and quality of lentiviral vector stocks. To achieve this various assays have been developed, which have been reviewed in depth by Delenda and Gaillard (2005), and more recently in brief by Ansorge *et al.* (2010). Those techniques most commonly employed are outlined here. Methods that directly measure vector components within supernatants are typically the fastest to perform. Such assays typically quantify either: (i) viral RNA by one-step reverse transcriptase (RT) quantitative PCR (RT qPCR) (Rohll *et al.*, 2002; Sastry *et al.*, 2002; Lizée *et al.*, 2003; Geraerts *et al.*, 2006), (ii) RT activity using the product enhanced reverse transcriptase (PERT) method (Rohll *et al.*, 2002), or (iii) the p24 CA protein of HIV-1 using an enzyme-linked immunosorbent assay (ELISA) (Geraerts *et al.*, 2006). These techniques do not discriminate between functional (active) and non-functional (inactive) lentiviral particles, but are used to assess total particle numbers. They are primarily employed during early process development studies or are utilised in conjunction with functional titre assays to assess the overall quality of lentiviral vector stocks (Segura *et al.*, 2006; Ansorge *et al.*, 2010).

Functional titre assays are conducted after the transduction of target cells. Transduction efficiency is cell line dependent (Ikeda *et al.*, 2002). For example, HEK293 were found to be superior to HeLa (human cervical carcinoma) and *Mus dunni* (murine fibroblast) cell lines (Sastry *et al.*, 2002), but inferior to NIH 3T3 (murine embryonic fibroblast), HEK293T and FRL 19 (rat liver carcinoma) cell lines (Zhang *et al.*, 2004). Zhang *et al.* (2004) also reported that the number of target cells, volume of vector, vector stability and adsorption period impacts on transduction efficiencies, and varying these parameters (as well as the cell type) caused more than 50-fold differences in titre. Using controlled transduction conditions is clearly important if results from different assays are to be meaningfully compared. Unfortunately no single procedure, or even assay standard, is widely used, making it difficult to compare results published by different groups. Following the transduction of target cells, the following parameters are commonly quantified (i) proviral DNA by qPCR (Rohll *et al.*, 2002; Sastry *et al.*, 2002; Lizée *et al.*, 2003), (ii) cellular mRNA using one-step RT qPCR (Lizée *et al.*, 2003; Geraerts *et al.*, 2006), or (iii) expression of a target protein or reporter gene, e.g. cells

expressing GFP are enumerated using flow cytometry (Sastry *et al.*, 2002; Lizée *et al.*, 2003; Geraerts *et al.*, 2006).

Assays other than those described here may be used to qualify the safety of lentiviral vector batches for clinical release (e.g. to demonstrate a lack of RCL, minimal levels of endotoxin etc.). These assays are not commonly performed during bioprocess development studies, and will not be covered here. Up-to-date information on this topic maybe found in the recent review by Segura *et al.* (2013).

1.4.5 Challenges in the development of an industrially viable bioprocess for ProSavin®

ProSavin® is a lentiviral vector engineered for treatment of Parkinson's disease, for which Phase I/II clinical trials have been successfully completed (Section 1.3.3). These trials required relatively small amounts of vector material, which was generated by transiently transfecting adherent monolayers of HEK293T with three plasmids (an overview of the process is provided in Table 1.7). However, for Phase III clinical trials and beyond, the use of stable producer cells that are amenable to growth in suspension is highly desirable (Sections 1.4.1 and 1.4.2). As a first step towards this goal, two tet-ON inducible stable ProSavin® producer cell lines that yield comparable titres to the standard HEK293T transfection protocol have been developed (Stewart *et al.*, 2009; 2011). One of these cell lines, termed PS46.2, was successfully adapted to growth in suspension (Stevenson *et al.*, 2009). However, while PS46.2 yields titres of 4.4 x 10^5 TU mL^{-1} when cultured adherently (Stewart *et al.*, 2009), in suspension mode titres were diminished 5- to 20- fold (K. Mitrophanous, pers. comm.). The reason for this drop in titre is unknown: cell aggregation in suspension cultures (Merten *et al.*, 2001), changes in the morphology and membrane properties of producer cells during suspension adaption (Ansorge *et al.*, 2010), and non-optimised culture conditions (Segura *et al.*, 2013) may all play a role in reducing titres. For clinical applications, crude titres of at least 2 x 10^5 TU mL^{-1}, equalling those attained using the current HEK293T transfection protocol (Schweizer and Merten, 2010), are required. Compensating for lower crude titres by altering the downstream process is both unfeasible (supernatants are already concentrated 2000-fold in two ultrafiltration steps, see Table 1.10) and undesirable, as the final product would comprise a greater proportion of impurities. Thus at present, use of suspension-adapted PS46.2 is not a viable option for the future manufacture of

ProSavin®. Lack of a scalable upstream process for ProSavin® could impede its future commercialisation. It is therefore vital that this issue is addressed. As a first step, a better understanding of the factors influencing lentiviral vector yields from suspension cultures of HEK-derived producer cells is required. As outlined below, this can most rapidly be achieved using a combination of microscale experimentation and statistical Design of Experiments (DoE) techniques.

1.5 Approaches to upstream process development

A wide range of parameters may affect lentiviral vector production from suspension-adapted producer cells. In order to characterise their various effects, numerous experiments are required. A rational approach combining microscale bioprocessing and DoE techniques can simplify this process by enabling rapid, low-cost, parallel, multi-parameter experimentation. The utility of such an approach has been demonstrated for the optimisation of protein expression from microbial cultures (Islam *et al.*, 2007; Holmes *et al.*, 2009). It was recently hypothesised that application of DoE to lentiviral vector systems would yield more efficient bioprocesses and better characterised products (Segura *et al.*, 2013) however, to the best of my knowledge no studies have employed this methodology, or tested the value of a microscale platform, to date. This section provides an overview of currently available small-scale culture systems and gives an introduction to DoE techniques, with a view to their application for the characterisation of lentiviral vector production from suspension-adapted producer cells. As these tools are only useful if they generate data that is relevant upon scale-up to larger vessels, the section concludes by considering which criteria may be appropriate for scale translation.

1.5.1 Small-scale culture systems

Microscale bioprocessing enables numerous parameters to be characterised rapidly in parallel and at low cost (Kumar *et al.*, 2004; Micheletti and Lye, 2006; Lye *et al.*, 2009; Marques *et al.*, 2010; Neubauer *et al.*, 2013). Shake flasks, with fill volumes of 10 - 500 mL, are the classic small-scale culture vessel, having been employed in process development studies for over fifty years (Betts and Baganz, 2006). Shake flasks are cheap and simple to use, requiring only an orbital shaking platform for their operation (Büchs, 2001a; Kumar *et al.*, 2004). Oxygen limitation is the most commonly cited problem when using shake flasks for microbial cultures

(Büchs, 2001a), however this is not generally a concern for mammalian cell cultures, whose oxygen demands are considerably lower (Micheletti *et al.*, 2006). Shake flasks are not typically instrumented (Kumar *et al.*, 2004), thus fermentation parameters are not monitored or controlled as they would be at large-scale leading to an absence of quantitative data (Neubauer *et al.*, 2013). Another drawback of shake flasks is their bulkiness, for example just twelve 250 mL flasks (50 mL typical working volume) will fit on a standard IKA KS 260 (Sigma-Aldrich) shaking platform (own observations). Their geometry also makes them unsuited to automation (Lye *et al.*, 2003), thus overall their utility for high throughput experimentation is somewhat limited.

Like shake flasks, orbitally shaken microwell plates are also low-cost and easy to use, however they require less space and culture materials, and are amenable to automation; thus they facilitate a greater degree of parallelisation (Kumar *et al.*, 2004; Neubauer *et al.*, 2013). Plates with 6, 12, 24 or 96 wells operating with a fill volume of 20 - 2000 µL are most commonly employed (Lye *et al.*, 2003; Micheletti and Lye, 2006). A range of well geometries and construction materials are available (Lye *et al.*, 2003; Betts and Baganz, 2006). Although plates are generally produced in plastic (thus are single-use), glass and metal formats also exist (Lye *et al.*, 2003; Betts and Baganz, 2006). Like shake flasks, microwell plates rely on surface aeration therefore have a limited oxygen transfer capacity (Betts and Baganz, 2006). However, the highest oxygen mass transfer coefficient (k_La) value reported for shaken plates is 792 hr^{-1} (Micheletti and Lye, 2006), which should support most microbial cultures, including yeast and *Escherichia coli* for example, whose typical k_La requirements are reported as 200 - 400 hr^{-1} (Bareither and Pollard, 2011). For mammalian cells, a k_La of just 1 hr^{-1} is generally thought sufficient to support the oxygen demand of 10^6 - 10^7 cells mL^{-1} (Barrett *et al.*, 2010; Bareither and Pollard, 2011). Consequently, oxygen limitation is unlikely to be a concern when using shaken microwells to culture mammalian cells. Perhaps not surprisingly therefore, shaken microwells have so far been successfully applied to the cultivation of HEK293, Chinese hamster ovary (CHO), and murine hybridoma cell lines (Girard *et al.*, 2001; Deshpande *et al.*, 2004; Davies *et al.*, 2005; Micheletti *et al.*, 2006; Barrett *et al.*, 2010; Silk *et al.*, 2010). Like shake flasks, microwell plates are not commonly instrumented, although versions with integrated sensors for pH measurements (Weiss *et al.*, 2002; Elmahdi *et al.*, 2003) and dissolved oxygen readings (Stitt *et al.*, 2002; John *et al.*, 2003; Deshpande *et al.*, 2004) have been developed.

PreSens Precision Sensing GmBH have also created a set of non-invasive optical sensor spots for pH, oxygen and carbon dioxide monitoring which can be applied to most microwell geometries. A drawback of using microwell plates is the risk of cross-contamination from aerosols formed at fast shaking speeds (Kumar *et al.*, 2004), however this is more of an issue for microbial systems that require high agitation rates to maintain adequate oxygen transfer. For mammalian cells, which are relatively much slower growing, cultures can last for several days and evaporation instead becomes a concern (Girard *et al.*, 2001; Deshpande *et al.*, 2004). However, this can largely be overcome by using a humidified incubator (Deshpande *et al.*, 2004) and a suitable plate cover (Micheletti *et al.*, 2006; Barrett *et al.*, 2010; Silk *et al.*, 2010), by filling the intersections of wells with sterile water (Girard *et al.*, 2001), and by feeding the cultures with additional water (Deshpande *et al.*, 2004) or medium (Silk *et al.*, 2010).

Minibioreactors, which are essentially small replicas of conventional stirred bioreactors, have been developed as an alternative to shaken systems for early bioprocess development studies (Kumar *et al.*, 2004; Betts and Baganz, 2006; Bareither and Pollard, 2011). These systems typically have a capacity of 50 - 300 mL, and allow temperature, pH, and dissolved oxygen to be monitored and controlled (Kumar *et al.*, 2004). As they are similar to stirred bioreactors in terms of their geometry and means of aeration and agitation, scale-up should be relatively straightforward (Kim *et al.*, 2012). Minibioreactors were initially developed for microbial cultures (Kostov *et al.*, 2001; Lamping *et al.*, 2003; Zanzotto *et al.*, 2004), and applications to mammalian cell culture are somewhat lagging (Ge *et al.*, 2006). Although several commercialised platforms are available, for example the ambr™ micro bioreactor from TAP Biosystems and the Micro-24 MicroReactor from Pall Corporation, the exorbitantly high cost of these systems, and their lack of utility for high-throughput experimentation (Kumar *et al.*, 2004), has likely limited uptake in this area.

1.5.2 Design of Experiments (DoE)

One-factor-at-a-time (OFAT) experiments, in which a single factor is varied while all others are fixed, are all too commonly employed when developing and optimising manufacturing processes (Czitrom, 1999). An OFAT approach entails numerous experiments, but generates little information about the system under investigation (Tye, 2004). The approach assumes that all

factors independently influence the measured response, thus the optimal setting for one factor stays constant regardless of variations in the other factor settings (Islam *et al.*, 2007). With regards to complex biological systems, this presumption is likely over-simplistic, and as a result the true optimal operating conditions may fail to be identified (Islam *et al.*, 2007). Multi-parameter experimentation using a structured DoE approach, on the other hand, generates more precise information from a smaller set of experiments that are linked in a rational manner (Tye, 2004). The fundamental advantages of undertaking a DoE approach, as compared to OFAT experimentation, are that it: (i) is less resource-intensive, (ii) more precisely estimates the impact of each factor, (iii) accounts for factor interactions, and (iv) generates experimental data for a larger region of the design space (Czitrom, 1999).

The first stage in implementing DoE is to define the experimental objective (Figure 1.4; Eriksson *et al.* (2008)). For early stage bioprocess development studies this will likely be *screening,* initially, to identify the key parameters impacting on product quality, followed by *optimisation* to further characterise the precise effect of those parameters and ascertain their optimum levels - the outcome of which should inform subsequent scale-up. Decisions made throughout the succeeding steps of the DoE process (outlined in Figure 1.5) are largely governed by the choice of experimental objective. Once the objective has been defined the factors to be investigated should be specified and given an experimental range, and responses chosen (e.g. for lentiviral vectors this would likely include titre). A regression model, based on either a linear, interaction or quadratic polynomial model, is next selected and a design generated according to the choice of model. Most commonly, regularly arranged factorial or central composite designs are employed (Eriksson *et al.*, 2008). Factorial designs comprise two levels per factor, thus can only support linear and interaction models, limiting their application to *screening*; central composite designs, on the other hand, can support quadratic models as each factor is explored over three or five levels, making them suitable for *optimisation* (Eriksson *et al.*, 2008). The final step prior to data collection is the generation of a worksheet that details the specific factor levels to be employed during individual experimental runs.

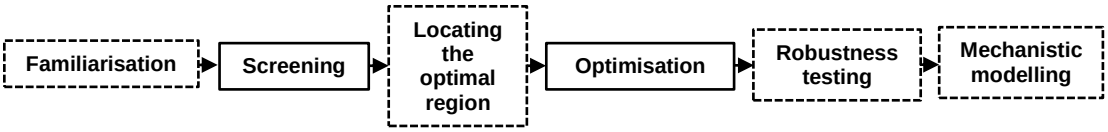


Figure 1.4 Experimental objectives used to direct Design of Experiments (DoE). A *familiarisation* study is usually only performed if researchers are confronted with an entirely new experimental system - minimal resources are expended to determine what is experimentally feasible. *Screening* experiments examine many factors in little depth, the objective being to discover which factors are most influential and their approximate optimum ranges. Occasionally this is followed by a second experiment to *locate the optimal region*, if the optimum did not seem to be contained within the experimental region explored during *screening*. *Optimisation* experiments are conducted to gain detailed information about the relationship between a few critical factors and the measured response, and to identify optimum settings for these factors. *Robustness testing* is commonly only carried out before a product or process is released, the aim being to uncover all parameters that may influence the result and regulate them such that the outcome remains within specifications. *Mechanistic modelling* is only relevant if a theoretical model is to be established. Information derived from Eriksson *et al.* (2008).

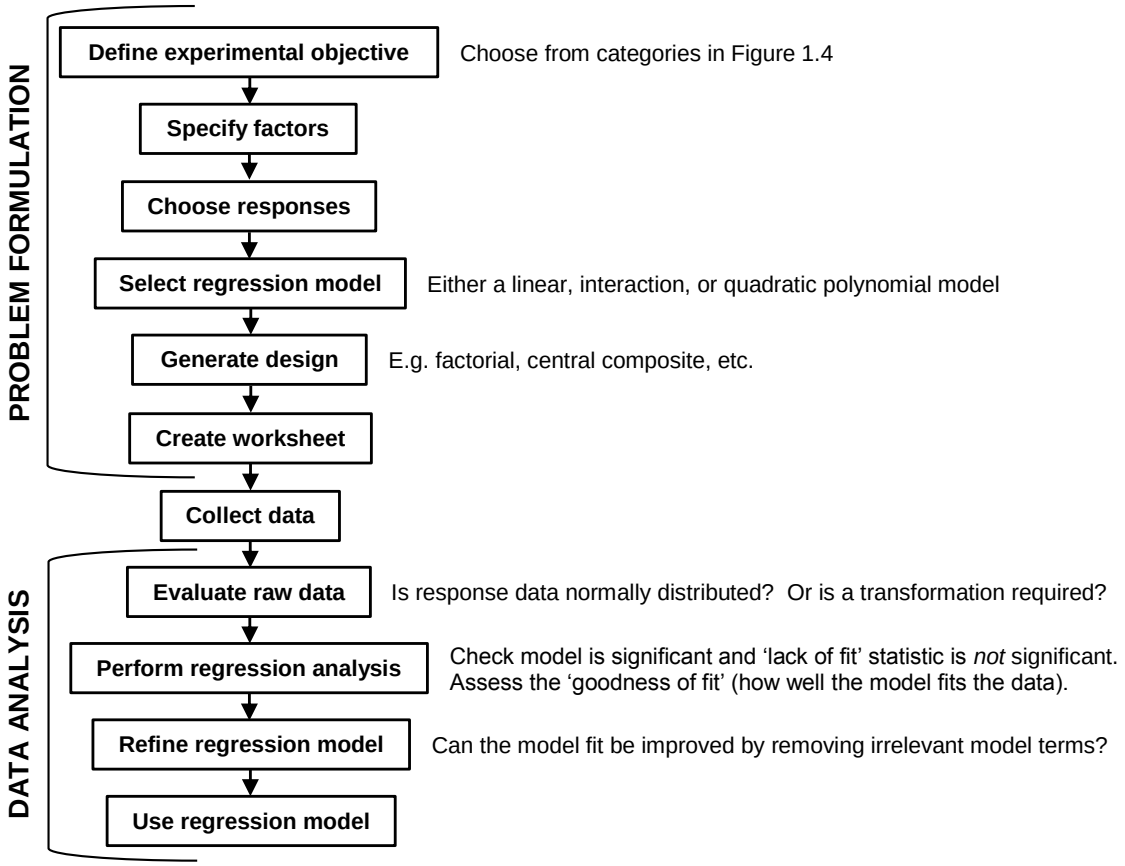


Figure 1.5 Common stages of DoE. Information derived from Eriksson *et al.* (2008).

Once the raw data has been collected, it should be evaluated to determine its suitability for regression analysis- a range of plots are available to help at this stage (Anderson and Whitcomb, 2007; Eriksson *et al.*, 2008). Of particular utility are the 'Normal' plot and 'Box-Cox' plot, which reveal if response data is approximately normally distributed and, if not, which transformation should be applied (Anderson and Whitcomb, 2007). Once satisfied that the data is suitable for further analysis, regression analysis can be carried out. The 'goodness of fit' and ANOVA statistics should subsequently be scrutinised. The 'goodness of fit' statistics comprise the adjusted R^2 ($adjR^2$) and predicted R^2 ($predR^2$) values, which together indicate how well the regression model fits the experimental data (Eriksson *et al.*, 2008). The $adjR^2$ (which has a value between zero and one) and the $predR^2$ (which may be any value from minus infinity to one) indicate the proportion of the response variation explained and predicted by the model, respectively (Islam *et al.*, 2007; Eriksson *et al.*, 2008). The model fit is generally thought adequate if both values are high (for $predR^2 \geq 0.5$ is good, while ≥ 0.9 is excellent) and ideally not separated by a value of more than 0.3, although the limits deemed acceptable will obviously vary depending on the application (Eriksson *et al.*, 2008). The ANOVA statistics are used to determine if a given model is significant, and whether it is valid based on a comparison between the calculated replicate error and model error - low model error is desired and is denoted by a 'lack of fit' *p* value that is above 0.05 (Anderson and Whitcomb, 2007; Eriksson *et al.*, 2008). If a model contains irrelevant terms, i.e. terms which do not significantly impact on the response, it is logical to explore the consequence of removing these, as refining the model in this way can improve the 'goodness of fit' (Eriksson *et al.*, 2008). The final step is to use the model. If the objective was *screening*, plotting the regression coefficients (with 95 % confidence intervals) can aid quick identification of those factors exerting the greatest impact on the measured response; if the objective was *optimisation*, a response surface plot can be useful for locating the optimal operating region (Islam *et al.*, 2007). The DoE approach specifically employed in this research project is described further in Chapter 2.

Finally, it is worth recognising the difficulties in applying DoE techniques to complex biological systems. The presence of uncontrolled factors or mechanisms can lead to high model error (Islam *et al.*, 2007), while variability between replicate response measurements can cause high experimental error, both of which can contribute to a poor fitting model. Indeed, reducing

variability (rather than increasing yield) may in fact be the main goal of optimisation studies when using biological systems (Tye, 2004). The occurrence of interactions between controlled factors can also compromise the accuracy of a model if not accounted for due to limitations in the chosen design (fractional factorial designs, for example, do not support the analysis of factor interactions).

1.5.3 Criteria for scale translation

Microscale cell culture systems can only support the rapid development and optimisation of bioprocesses if the data they generate is reliably scalable (Micheletti *et al.*, 2006; Marques *et al.*, 2010). Although scale-up studies are therefore critical if a microscale platform is to be considered of any worth, surprisingly little has been published on the subject (Micheletti *et al.*, 2006). This is probably because no straightforward or universal strategy exists for tackling the matter (Betts and Baganz, 2006; Marques *et al.*, 2010). For every new system, key process parameters likely to affect product quality need timely identification and characterisation (Betts and Baganz, 2006; Marques *et al.*, 2010). Those conditions producing optimum results in the small-scale vessel should be maintained as far as possible during scale-up (Doran, 1995; Marques *et al.*, 2010). Biological and chemical parameters (such as cell density, concentration of inducer compounds etc.) are often relatively straightforward to preserve at constant levels. Replicating the physical culture environment presents a much greater challenge, however, and to achieve successful scale-up an understanding the mass transfer and fluid mixing properties of both the small- and large-scale vessels is critical. To date, scale-up from flasks, microwells or minibioreactors has been most often performed on the basis of matched oxygen mass transfer coefficient (k_La) values for microbial systems (Marques *et al.*, 2010), while mean energy dissipation rates (power per unit volume; *P/V*) or mixing times have been used as scale-up criteria for mammalian cell cultures (Micheletti *et al.*, 2006; Barrett *et al.*, 2010; Gill *et al.*, 2011).

Early scale translation studies have focused on shifting bacterial processes into stirred tank bioreactors. For this application, k_La has proven to be the most appropriate scaling criterion (Kostov *et al.*, 2001; Micheletti *et al.*, 2006; Gill *et al.*, 2008; Islam *et al.*, 2008; Zhang *et al.*, 2008; Marques *et al.*, 2009). Broadly, k_La describes the capacity of a culture vessel to deliver oxygen to cells (Doran, 1995). Specifically, it characterises the transfer of oxygen from gas to

liquid, the driving force being the gradient between the oxygen concentration at the gas-liquid interface and the average oxygen concentration in the bulk liquid (Garcia-Ochoa and Gomez, 2009). k_La is affected by both gas solubility (which in turn is influenced by temperature, pressure, media composition, and chemical reactions) and the metabolic activity of the cells, and may be determined experimentally or estimated using empirical correlations - the former method being generally deemed the most accurate (Garcia-Ochoa and Gomez, 2009). k_La is likely the most useful scaling criterion for bacterial processes because the oxygen uptake rate for bacterial cells is extremely high – around 0.46 - 2.33 x 10^{-3} g $cell^{-1}$ hr^{-1} (Micheletti *et al.*, 2006) – and maintaining a ready supply of dissolved oxygen is therefore crucial to avoid sub-optimal oxygen limited conditions (Marques *et al.*, 2010).

Unlike bacteria, the oxygen uptake rate for mammalian cells is several orders of magnitude lower – approximately 1 - 10 x 10^{-12} g $cell^{-1}$ hr^{-1} – thus oxygen transfer is not commonly considered a limiting factor for cell growth (Micheletti *et al.*, 2006). Use of k_La as a scaling factor for mammalian cell cultures is therefore less appropriate. As mammalian cells lack a cell wall and are consequently more sensitive to shear forces than bacteria (Kretzmer and Schügerl, 1991), the hydrodynamic forces within the culture vessel instead require important consideration. Shear sensitivity is governed by cell type, the extent of vorticity around the cell, the proximity of the cell to solid-liquid and gas-liquid interfaces (bubbles rising through the liquid and bursting at the surface can contribute to cell damage), and local hydrodynamic energy dissipation (Doran, 1995; Heath and Kiss, 2007). Owing to its direct relation to shear rate, energy dissipation rate, or *P/V*, has been evaluated as a criterion for scale-up of antibody production processes based on murine hybridoma cell cultures (Micheletti *et al.*, 2006; Barrett *et al.*, 2010). The average *P/V* in this instance was determined for microwells using computational fluid dynamics (CFD) simulations, for shake flasks using the correlation of Büchs *et al.* (2000a), and for a stirred bioreactor by direct measurement of the impeller power number (Barrett *et al.*, 2010). Microwell and shake flask fermentations were subsequently performed based on an average *P/V* value of 40 W m^{-3}, however a lower average *P/V* value of 3.64 or 8 W m^{-3} had to be accepted for stirred bioreactor fermentations (Micheletti *et al.*, 2006; Barrett *et al.*, 2010). Although operation of the stirred bioreactor at matched *P/V* was desired, it was unfeasible due to the high impeller speed required to attain the average value of 40 W m^{-3} - when trialled

excessive foaming had resulted (Barrett *et al.*, 2010). Nevertheless, despite the lower *P/V* values in the stirred bioreactor, similar cell growth and antibody production kinetics were observed for all three vessel types (Micheletti *et al.*, 2006; Barrett *et al.*, 2010). However, as the *P/V* in the stirred bioreactor could not be matched to that of the microwell or shake flask, Micheletti *et al.* (2006) concluded that *P/V* was useful as a basis of scale translation between shaken vessels only. It is worth noting that most cell damage in stirred bioreactors is highly localised around the impeller zone, therefore an impeller zone *P/V* (rather than average *P/V*) may be useful in the future for scale-up between stirred vessels.

Scale-up from shaken vessels to a WAVE bioreactor based on matched *P/V* could be feasible, although this has not been trialled. The average *P/V* for a 2 L WAVE bioreactor, calculated from film sequences that had been analysed using Computer Aided Design (CAD), was recently reported to be between 8 and 561 W m^{-3}, depending on the fill volume and rocking conditions used (Eibl *et al.*, 2010b). It is therefore conceivable that the average *P/V* associated with optimal culture conditions in small shaken vessels may be replicated in a WAVE system, although the same degree of hydrodynamic stress, or cell culture performance would not necessarily be guaranteed. The mode of mixing differs markedly between small shaken vessels and wave-mixed systems, leading to different local and temporal energy dissipation rates. For example, Barrett *et al.* (2010) reported a maximum local *P/V* of 2 kW m^{-3} (observed at the gas-liquid interface) for microwells operating under standard cell culture conditions. For a 2 L WAVE bioreactor (with 1 L fill volume), CFD simulations revealed that during a single rock the average *P/V* value peaks and drops two or four times, fluctuating between zero and approximately double the overall average (Eibl *et al.*, 2009). Knowledge of the maximum *P/V* likely to be encountered within a vessel is important, as high values may compromise culture performance. For example, a value of 1 kW m^{-3} was found to detach CHO cells from microcarriers, while values in the range of 1000 kW m^{-3} produced a cessation in cell growth and gradual decline in cell viability (Heath and Kiss, 2007). Cell types obviously differ in their susceptibility to shear damage, however, but of note is the fact that clusters of cells, along with cells attached to microcarriers, are likely to be most vulnerable (Mollet *et al.*, 2004).

Interestingly, although the feasibility of *P/V* as a basis for scale-up into WAVE bioreactors has not been tested, scale-up from microwells to a WAVE system has been successfully performed

on the basis of mixing time (Gill *et al.*, 2011). Mixing time may be defined as: "the time required to achieve a given degree of homogeneity starting from the completely segregated state" (Doran, 1995, p. 147). Mixing time may be considered a function of *P/V*, as to decrease mixing time an increase in *P/V* is usually required. It is often measured by the addition of sodium thiosulphate to an iodine solution under standard vessel fill volume and agitation conditions - the time taken for complete (or near complete) decolourisation to occur is then recorded (Nienow *et al.*, 1996). Mixing time may thus be assessed relatively simply regardless of the geometry or instrumentation capabilities of a particular vessel. In this sense it is therefore an attractive scaling parameter, however it has rarely been employed as such for stirred bioreactors, as maintaining a constant mixing time when transferring the process to larger vessels would demand a considerable increase in *P/V*, which could not feasibly be realised with most stirring equipment (Doran, 1995). In the study by Gill *et al.* (2011), an antibody production process based on CHO cell culture was successfully scaled from a shaken microwell to a WAVE bioreactor using a relatively fast mixing time of around 5 s. Mixing time could therefore be a useful basis of scale translation between small shaken vessels and wave-mixed systems.

Dimensionless numbers, although not useable as scaling parameters in themselves, can also provide insight into the mixing environment within small scale culture vessels, which in turn can inform the choice of operating conditions during scale-up. Particularly useful are the Reynolds number (*Re*), Froude number (*Fr*) and Phase number (*Ph*). *Re* is a parameter that characterises the relative importance of inertial and viscous effects, and can be used to determine whether fluid flow is laminar, creating a non-homogenous environment, or turbulent, creating a well-mixed environment (Happel and Brenner, 1983; Doran, 1995). *Re* can be calculated for shaken systems using the equation of Büchs *et al.* (2000b), although only one study has attempted to link calculated values to observed flow regimes - in this instance for a 24-well plate culture format (Barrett *et al.*, 2010). *Fr* is a ratio of inertial and gravitational forces (Doig *et al.*, 2005) that is used to establish the relative influence of gravity on fluid hydrodynamics. Thus, *Fr* could be used to ascertain whether the culture environment encourages the settling of cells due to the influence of gravity. When considered in conjunction with the *Fr*, *Ph* can be used to describe whether the bulk of a liquid in a shaken vessel is circulating "in-phase" with the shaking platform, or whether so-called "out-of-phase" conditions

are present such that the majority of the liquid remains stationary in the centre of the vessel and only a minor fraction is actually rotating along the vessel wall (Büchs *et al.*, 2001b; Barrett *et al.*, 2010). *Fr* may be calculated for shaken systems using the equation of Büchs *et al.* (2000b), while *Ph* may be calculated using the equation of Büchs *et al.* (2001b). Only for conditions where the *Fr* > 0.4 and the *Ph* > 1.26 can the influence of gravity be disregarded and "in-phase" operation be assumed (Büchs *et al.*, 2000b; Barrett *et al.*, 2010). "In-phase" conditions are associated with better mixing and gas transfer.

With regard to lentiviral vector process development, it is acknowledged that bioreactor cell culture conditions may profoundly impact on titres, and optimal conditions are generally cell line and lentiviral vector-specific (Segura *et al.*, 2013). It is evident that the field would benefit from the development of a generic microscale platform that is suitable for the culture of suspension-adapted HEK293-derived cell lines, and that can be employed for the generation of reliably scalable bioprocess design data.

1.6 Thesis aims and objectives

Despite concerns that current lentiviral vector manufacturing practices, based on transient transfection of adherent HEK293(T) cells, cannot be easily scaled to meet future commercial requirements, alternative production strategies based on suspension cell culture appear to have been little explored (Segura *et al.*, 2007; Broussau *et al.*, 2008; Ansorge *et al.*, 2009; Witting *et al.*, 2012). To support the design of a simple, scalable manufacturing route for lentiviral vectors, the overall aim of this thesis is to establish a microwell experimental platform based on the suspension culture of stable producer cell lines that can be employed for the rapid, early generation of relevant bioprocess design data. The approach will be illustrated using ProSavin®, a lentiviral vector for treatment of Parkinson's disease that has recently completed a Phase I/II clinical trial (Mitrophanous *et al.*, 1999; Azzouz *et al.*, 2002; Jarraya *et al.*, 2009; Stewart *et al.*, 2009; Stewart *et al.*, 2011; Palfi *et al.*, 2014). The specific objectives of this thesis are:

- To establish microwell methods for the suspension culture of HEK293T-derived producer cells, and to evaluate the utility of the microscale platform (in conjunction with statistical DoE techniques) for the rapid screening of several operating parameters to

ascertain their relative impact on lentiviral vector titre. This work is described in Chapter 3.

- To determine whether a DoE-guided microwell approach is efficient in optimising the yield and quality of lentiviral vector preparations. This work is described in Chapter 4.
- To discover whether the insights gained during microwell experiments can inform laboratory scale operation of a single-use WAVE bioreactor system. This work is described in Chapter 5.
- To ascertain the half-life of lentiviral vectors under process-relevant conditions and consider how this knowledge may be applied to further improve bioprocess design. This work is described in Chapter 6.

In addition, Appendix A outlines the hypothetical next steps that would be required to validate the production approach that is presented in Chapter 5. Finally, Chapter 7 contains overall conclusions from this study and suggestions for future work.

2 Materials and methods

2.1 General cell culture methods

2.1.1 PS46.2 cell line

A stable ProSavin® producer cell line, PS46.2, that had previously been adapted to growth in suspension was utilised for the majority of work described herein. PS46.2 is a human embryonic kidney (HEK)293T cell line that has been stably transfected with the four plasmids required to create a ProSavin® producer cell line (Stewart *et al.*, 2009; 2011). PS46.2 constitutively expresses the vector genome, but expression of both packaging components (vesicular stomatitis virus envelope protein (VSV-G) and EIAV Gag/Pol) is controlled by the tetracycline (tet) repressor (TetR) regulatory protein. Expression of VSV-G and Gag/Pol is initiated by addition of doxycycline (dox; Sigma-Aldrich, Poole, UK), an analogue of tet, to the culture medium. Adaption of PS46.2 to suspension growth mode was carried out by L. McCloskey (Oxford BioMedica, Oxford, UK) using a method of gradual serum reduction as described in Guy *et al.* (2013). Suspension-adapted PS46.2 were kindly supplied by either L. McCloskey or S. Denby (Oxford BioMedica). Cells provided by S. Denby were utilised for the initial shake flask and microwell studies described in Sections 3.2.1 and 3.2.2. Cells obtained from L. McCloskey were used to generate working cell banks as described in Section 2.1.5; these stocks supplied all other suspension-based PS46.2 studies. PS46.2 that had *not* been adapted to growth in suspension were required for the stability studies described in Chapter 6, these cells were kindly provided by H. Stewart (Oxford BioMedica).

2.1.2 HEK293T cell line

Adherent HEK293T (Stanford University, Stanford, CA, USA) were utilised for the production of lentiviral vectors by transient transfection (Section 2.10.3) and for titration of lentiviral vectors by either the rapid ProSavin® titre assay (Section 2.11.2.1) or the DNA integration assay (Section 2.11.2.3). Early-passage adherent HEK293T cells were obtained from the Research/PAR Group at Oxford BioMedica. Suspension-adapted HEK293T (obtained from S. Denby and originally derived from the Stanford University adherent cell stock) were used for the shake flask *versus* microwell comparative study described in Section 3.2.3.

2.1.3 D17 cell line

For titration of lentiviral vectors expressing the *LacZ* marker gene (Section 2.11.2.2), adherent canine (D17) cells (ECACC, Salisbury, UK; Catalogue number: 89090403) were used. Early-passage D17 were obtained from the Research/PAR Group at Oxford BioMedica.

2.1.4 Culture media

Suspension-adapted PS46.2 and suspension-adapted HEK293T were maintained in Freestyle™ 293 expression medium (Invitrogen, Paisley, UK) supplemented with 1 - 5 % (v/v) tet-free foetal calf serum (FCS; PAA Laboratories, Yeovil, UK). Adherent PS46.2 were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) supplemented with 2 mM L-glutamine (Sigma-Aldrich), 1 % (v/v) nonessential amino acids (Sigma-Aldrich) and 10 % (v/v) tet-free FCS. Adherent HEK293T and D17 cells were maintained in DMEM supplemented with 2 mM L-glutamine, 1 % (v/v) nonessential amino acids and 10 % (v/v) FCS (*not* tet-free; Lonza, Basal, Switzerland).

Prior to use in cell culture, all FCS was heat-inactivated to reduce any complement factors and adventitious agents that may be present. This was an extra precaution as FCS is gamma-irradiated and tested by the supplier for various adventitious agents prior to its release for cell culture purposes. FCS was heat-inactivated in a water bath set to 56 °C for 30 min, then aliquoted and stored at -20 °C until required.

It is important to highlight that selection antibiotics were *not* added to the culture medium for any of the work described herein. PS46.2 cells provided by L. McCloskey and H. Stewart had been previously cultured in the presence of the four antibiotics used for selection of PS46.2 (Stewart *et al.*, 2009) to prevent loss of vector components. However, stability of the vector components in the absence of selection antibiotics was demonstrated over multiple passages (up to 111 days) for adherent PS46.2 by Stewart *et al.* (2011). In addition, L. McCloskey tested functional vector production from suspension-adapted PS46.2 periodically over a 56 day period, and found titres to remain consistent over this timeframe also in the absence of antibiotic selection (Guy *et al.*, 2013). Thus for the work described herein, selection antibiotics were omitted from the culture medium in order to reduce process complexity. As a precaution, PS46.2 cells were not

passaged for periods longer than 56 days (inclusive of the time taken to generate cell banks as described in Section 2.1.5).

2.1.5 Creation of PS46.2 cell banks

Suspension-adapted PS46.2 that had been previously cryopreserved by L. McCloskey in culture medium containing 1 or 5 % (v/v) tet-free FCS were revived and expanded using methods outlined in Section 2.1.6. After 10 days, cell banks of both the 1 % and 5 % (v/v) tet-free FCS-containing cultures were generated. Additionally at this stage, cells that had been expanded in medium supplemented with 1 % (v/v) tet-free FCS were transferred into medium containing 3 % (v/v) tet-free FCS and cultured for a further 10 days before also being banked. To create cell banks, cultures with > 90 % cell viability were transferred into 50 mL centrifuge tubes (BD Falcon™ conical tubes; BD Biosciences, San José, CA, USA) and centrifuged for 5 min at 150 x *g*. Culture supernatant was removed and cells were re-suspended at a density of 1 x 10^7 viable cells mL^{-1} in growth medium supplemented with 10 % (v/v) dimethyl sulphoxide (DMSO; Sigma-Aldrich). 1 mL aliquots of cell suspension were transferred into sterile Nunc® cryovials (Fisher Scientific, Loughborough, UK) and stored at -85 °C in a Nalgene® Mr Frosty® freezing container (Fisher Scientific) for 5 days, after which cell stocks were transferred to -150 °C for long term storage. When stocks of the 5 % (v/v) tet-free FCS-containing cell bank were low, cells from one of the remaining vials were revived and expanded for 10 days before being banked in accordance with the procedures outlined above. This cell bank demonstrated comparable cell growth and vector production capabilities to the preceding cell bank, when evaluated in parallel. All cell banks passed in-house sterility and mycoplasma testing, which was carried out by the laboratory management team at Oxford BioMedica.

2.1.6 Revival of PS46.2 cell stocks and routine subculture

For revival of suspension-adapted PS46.2, one cryovial (containing ~ 1 x 10^7 cells) was retrieved from -150 °C storage and quickly thawed in a 37 °C water bath; following which one of two protocols was used. When reviving cells for the purpose of creating working cell banks and for studies described in Chapters 3, 4 and 6, the cell suspension was aseptically transferred to a 125 mL shake flask (polycarbonate Erlenmeyer flask with vent cap; Sigma-Aldrich) and 20 mL pre-warmed media directly added drop-wise. For work described in Chapter 5, cells were first

aseptically transferred to a sterile 50 mL centrifuge tube, where 20 mL pre-warmed media was added drop-wise. The tube was centrifuged for 5 min at 150 x *g*, after which the supernatant was removed. The cell pellet was resuspended in 20 mL pre-warmed media and finally transferred to a 125 mL shake flask. In the case of both revival methods, the shake flask was then placed inside a 36.5 °C humidified 5 % CO_2 incubator on a shaking platform with 10 mm orbital diameter (IKA KS 260; Sigma-Aldrich) rotating at 150 rpm. The centrifugation step was introduced as a precautionary measure to minimise residual DMSO in the culture medium, however when the different revival methods were evaluated in parallel, comparable cell recoveries were observed. For the maintenance of suspension-adapted PS46.2, shake flask cultures (250 mL polycarbonate Erlenmeyer flasks with vent caps, Sigma-Aldrich) at working volumes of 50 mL were routinely sub-cultured by dilution at 3 - 4 day intervals using a seeding density of 4 x 10^5 viable cells mL^{-1}. Cultures were kept on a shaking platform with 10 mm orbital diameter, rotating at a rate of 150 rpm, and were incubated at 36.5 °C in a 5 % CO_2 humidified incubator.

For revival of adherent PS46.2, two cryovials (each containing ~ 5 x 10^6 cells) were retrieved from -150 °C storage and quickly thawed in a 37 °C water bath. Cells were aseptically transferred to a single 50 mL centrifuge tube and 25 mL pre-warmed media was added drop-wise. The tube was centrifuged for 5 min at 150 x *g* and supernatant was removed. The cell pellet was resuspended in 25 mL pre-warmed media and transferred to a 75 cm^2 tissue culture flask (BD Falcon™ vented flask; BD Biosciences), and incubated at 36.5 °C in a humidified 5 % CO_2 incubator. Adherent PS46.2 were maintained in 150 cm^2 tissue culture flasks (BD Falcon™ vented flasks) containing 30 mL media, and were routinely sub-cultured at 3 - 4 day intervals using a seeding density of between 1.7 x 10^5 and 4.0 x 10^5 viable cells mL^{-1}. To passage cells, the culture medium was removed and cells were washed with 10 mL phosphate buffered saline (PBS; Sigma-Aldrich). 4 mL TrypLE™ Express dissociation agent (Invitrogen) was added to cells and the flask was incubated for 5 min at 36.5 °C to detach cells from the culture vessel. The dissociation agent was neutralised by addition of 6 mL media. Cells were then diluted to the correct seeding density using fresh media and transferred to a new tissue culture flask. Cultures were kept in a 5 % CO_2 humidified incubator at 36.5 °C.

2.2 Evaluation of a shaken microwell system for suspension culture of lentiviral vector producer cells

2.2.1 Growth kinetics and associated culture parameters for PS46.2 cultured in shake flasks and microwells

The growth kinetics and culture parameters of suspension-adapted PS46.2 grown in shake flasks *versus* microwells were compared (Section 3.2.1). Triplicate shake flasks (250 mL Erlenmeyer flasks with 50 mL working volume) and 18 wells of a microwell plate (standard 24 round well ultra-low attachment microwell plate; Corning Inc., Oneonta, NY, USA) were seeded in parallel using a density of 4 x 10^5 viable cells mL^{-1} in medium containing 5 % (v/v) tet-free FCS. Microwells were operated with an 800 µL working volume (total well capacity is 3400 µL) as, based on the earlier work by Barrett *et al.* (2010), this was predicted to deliver an adequate supply of oxygen for the growth of mammalian cells. Additionally, successful culture of both VPM 8 murine hybridoma (Micheletti *et al.*, 2006; Barrett *et al.*, 2010) and glutamine synthetase (GS)-CHO cell lines (Silk *et al.*, 2010) had previously been demonstrated when using an 800 µL fill volume in conjunction with this particular plate design. Microwell plates were sealed using a Duetz sandwich lid in conjunction with a metal clamp (EnzyScreen BV, Leiden, The Netherlands) as previously described (Duetz *et al.*, 2000; Silk *et al.*, 2010). The microwell system described here was subsequently utilised for all microscale studies included in this thesis. Sandwich lids were sterilised by autoclaving at 121 °C for 20 min and were dried in a drying cabinet (this was kindly performed by a member of the laboratory management team at Oxford BioMedica). Shake flasks and microwell plates were kept on an orbital shaking platform of 10 mm diameter (IKA KS 260; Sigma-Aldrich) rotating at a speed of 150 or 180 rpm, respectively. Cultures were maintained at 36.5 °C in a 5 % CO_2 humidified incubator. Sampling was conducted between 0 and 189 hr and various cell culture parameters (as listed in Sections 2.11.1.1 to 2.11.1.4) were monitored. To perform sampling at each time point, 1 mL culture was removed from each shake flask and triplicate wells of the microwell plate were sacrificed.

2.2.2 ProSavin® induction and harvest in shake flasks and microwells

ProSavin® titres from shake flask and microwell cultures of suspension-adapted PS46.2 were compared (Section 3.2.2). Shake flasks and microwell plates were initially set up as described in Section 2.2.1. These cells were induced 24 hr later by addition of 1.0 µg mL^{-1} dox and 10 mM sodium butyrate (NaBu; Sigma-Aldrich). Vector harvests were performed 24 hr post-induction. To harvest vector from shake flasks, supernatants were sterile filtered using syringe filters of 0.45 µm pore size (surfactant-free cellulose acetate membrane; Sartorius, Epsom, UK) and the resultant filtrate was stored in freezing vials (Sigma-Aldrich) at -80 °C. To harvest vector from microwell plates, supernatants were loaded on to a Multiscreen® HTS 96-well filter plate with 0.45 µm pore size (surfactant-free mixed cellulose ester membrane; Millipore; Fisher Scientific) and centrifuged for 5 min at 2050 x *g*. A 96-well U-bottom collection plate (BD Falcon™; BD Biosciences) was positioned underneath the filter plate during centrifugation. The collection plate was covered with sterile SealPlate film (Anachem Ltd, Luton, UK) and stored at -80 °C. Titres were quantified using the rapid ProSavin® titre assay (Section 2.11.2.1).

2.2.3 Growth kinetics and associated culture parameters for HEK293T cells cultured in shake flasks and microwells

The growth kinetics and culture parameters of suspension-adapted HEK293T cells grown in shake flasks *versus* microwells were compared (Section 3.2.3). The set-up for this experiment was identical to that described for the earlier evaluative study based on the PS46.2 cell line (Section 2.2.1). The only difference (other than the choice of cell line) was that the culture medium used in this study contained 1 %, rather than 5 %, (v/v) tet-free FCS.

2.3 Characterisation of shake flask and microwell liquid phase mixing times

The sodium thiosulphate method (Nienow *et al.*, 1996) was used to characterise mixing times in a variety of vessel sizes and geometries (Section 3.2.4). Mixing times were quantified in a microwell, Erlenmeyer shake flasks (125 mL, 250 mL and 500 mL sizes; Sigma-Aldrich) and an upright 2 L roller bottle (Corning®; Sigma-Aldrich) whilst maintaining their characteristic working

volume (0.8 mL for microwell plates; 20 % of total vessel volume for the shake flasks and roller bottle).

Briefly, the vessel was filled to 99 % of the working volume with a 5 mM iodine solution (Sigma-Aldrich), and securely mounted on an orbital shaking platform of 10 mm diameter. A metal clamp stand was also mounted on the shaking platform, and positioned such that a pipette could be held directly over the culture vessel. A pipette was then loaded with a volume of 1.8 M sodium thiosulphate (Sigma-Aldrich) that equated to 1 % (v/v) of the working volume. The platform was then switched on, and once the desired speed was reached (140, 180, 220 or 260 rpm for microwells; 150 rpm for the shake flasks and roller bottle), the sodium thiosulphate solution was dispensed into the vessel, and the time taken for complete decolourisation of the iodine solution recorded. Each mixing time experiment was performed in triplicate.

To visualise the mixing of small liquid additions made to bulk fluid within a shaken microwell (and reveal what would occur during cell culture e.g., during the addition of inducer compounds) the mixing time experiments described above were repeated using an inert food dye (Super Cook; Dr Oetker, Leeds, UK) in place of the sodium thiosulphate solution, and water in place of the iodine solution. Images were captured at a rate of 125 frames per second using a high speed Photron DVR video system (Photron Ltd, Tokyo, Japan; kindly loaned by the Engineering and Physical Sciences Research Council [EPSRC] Engineering Instrument Pool) mounted on the shaking platform. Images were obtained for shaking speeds of 140, 180 and 220 rpm, and a single flow visualisation experiment was performed for each condition.

2.4 Design of Experiments (DoE) methodology applied to the ProSavin®

2.4.1 Factorial design screening experiment

2.4.1.1 Experimental design

The influence of various operating parameters on ProSavin® production was investigated in microwell plates using a Design of Experiments (DoE) approach. Initially, the relative impact of nine factors (Table 3.2) on ProSavin® titres was assessed (Section 3.2.5). DoE software

(Design Expert version 8; Stat-Ease, Minneapolis, MN, USA) was used to create a resolution IV fractional factorial design, where each factor was varied over two levels (high and low) designed to perturb the experimental system. A full-factorial approach (covering all factor combinations) was not feasible, as this would equate to 512 experimental runs. By employing a resolution IV fractional factorial design, this was greatly reduced to 32 runs. Table 3.3 shows the experimental plan in which nine factors were screened in 32 experimental runs, plus four replicated centre point experiments were conducted for estimation of pure error (see Section 3.2.5.1). Experimental runs were carried out in triplicate using the microwell platform as described in Section 2.4.1.2, and titres were quantified using the rapid ProSavin® titre assay (Section 2.11.2.1).

It is important to note that, due to the requirement for different platform shaking speeds and diameters, the screening experiment was completed in several batches. Batch-to-batch variability is usually accounted for in DoE software by blocking the data (centre points are carried out alongside each batch so software can adjust the generated model according to the variability of repeated centre points). In this instance, however, centre points could not be carried out with every batch due to the use of a shaking platform with adjustable orbital diameter (i.e. centre points required a 20 mm diameter, while some runs required a 30 mm diameter; shaking platform was an SK-300 manufactured by Jeiotech, Seoul, Korea). To minimise the likelihood of uncontrolled batch-to-batch variability interfering with data analysis and interpretation, titres were normalised to (i.e. expressed as a percentage of) the titre of reference shake flask cultures run and assayed in parallel to each set of experiments. Shake flask procedures are described in Section 2.4.1.3.

For statistical calculations the levels of each variable were coded as described previously (Islam *et al.*, 2007), whereby the high level was coded as 1 and the low level was coded as -1. Using Design Expert software, a regression model was fitted to normalised titre data. A constant of 0.16 was added to titre values and the data was log transformed in order to render the distribution approximately normal. Only main terms that were significant at the level $p < 0.1$ were included in the model.

2.4.1.2 Microwell culture methods

Experimental runs were carried out using microwell cultures as described in Section 2.2.2, apart from the following modifications: (i) to seed microwells, suspension cultures of PS46.2 were first centrifuged for 5 min at 150 x *g*, after which the supernatant was removed, enabling the cells to be resuspended in completely fresh medium at the required density, (ii) to harvest ProSavin®, an additional centrifugation step (prior to filtration) was introduced to minimise filter clogging and maximise the volume of harvested vector - specifically, cultures were aliquoted into a 96-well U-bottom plate and centrifuged for 5 min at 2050 x *g*, after which supernatants were loaded in to a 96-well filter plate with 0.45 µm pore size and centrifuged for a further 5 min at 2050 x *g*, and (iii) freezing vials were used to store the collected filtrate at -80 °C. Specific operating conditions were as detailed in Table 3.3 (Section 3.2.5.1).

2.4.1.3 Reference shake flask culture methods

Parallel to microwell experiments, ProSavin® was produced using triplicate standard shake flask cultures of PS46.2. Again, this protocol is a revised version of that utilised earlier (Section 2.2.2). Here, an additional centrifugation step was introduced prior to seeding, which was carried out as described in Section 2.4.1.2. In addition, an extra centrifugation step (prior to filtration) was included to reduce filter clogging during ProSavin® harvests: cultures were transferred into 50 mL centrifuge tubes and centrifuged for 5 min at 2050 x *g*, after which supernatants were sterile filtered using syringe filters of 0.45 µm (the resultant filtrate was transferred into freezing vials and stored at -80 °C).

2.4.1.4 Photographs of cell aggregates

During the screening experiment it was noted that the cells aggregated, forming distinct arrangements that seemed dependent on the cell seeding density, serum content of the medium and mixing conditions of the microwells. To qualitatively capture this data, photographs were taken 46 hr subsequent to seeding using a FinePix F10 digital camera (Fujifilm, Düsseldorf, Germany) - see Section 3.2.5.2 and Figure 3.11 for details.

2.4.2 Three factor optimisation experiment with central composite face-centred (CCF) design

A central composite face-centred (CCF) experiment was conducted to identify approximate operating ranges for post-induction period, liquid fill volume and concentration of dox (Section 4.2.1.2). These factors were varied over the ranges 22 - 70 hr, 600 - 1000 µL, and 1.0 - 2.6 µg mL^{-1}, respectively, while all other factors were given a fixed value (Table 4.1). Table 4.2 shows the experimental plan for this experiment, in which the three factors were investigated in 20 runs, with six replicated centre point experiments included for estimation of pure error (see Section 4.2.1.2).

Experimental runs were carried out in triplicate using the microwell platform as described in Section 2.4.1.2, except that the values for post-induction period, liquid fill volume and concentration of dox were as detailed in Table 4.2, and all other operating conditions were fixed as detailed in Table 4.1. Cell growth, ProSavin® titre, and RNA copy number were quantified (methods described in Sections 2.11.1.1, 2.11.2.1, and 2.11.3.1), and the particle:infectivity (P:I) ratio was also calculated for each run, using the mean values for RNA copy number and titre (P:I ratio = RNA copy number / titre). ProSavin® titre and P:I ratio data was entered into Design Expert software and the behaviour of the system was modelled using a quadratic or two-factor interaction fit equation, respectively. An inverse transformation was applied to titre values, and a log transformation was applied to P:I ratio values, in order to render the distribution of each data set approximately normal. Model terms that were not significant at the level $p < 0.1$ were removed via backward elimination, starting with the least significant model terms, in order to maximise the resulting regression function of the model.

ProSavin® was produced using reference shake flask cultures, according to the protocol outlined in Section 2.4.1.3, parallel to this experiment, for the purpose of monitoring natural process variation only. It was not necessary to normalise microwell data to parallel shake flask data prior to analysis as was reported for the screening experiment (Section 2.4.1.1), as all experimental runs were carried out simultaneously as a single batch.

2.4.3 Further CCF experiment for two factor optimisation

To further refine the operating ranges for post-induction period and dox concentration, a second CCF experiment was conducted (Section 4.2.1.3). Post-induction period and concentration of dox were varied over the ranges 18 - 26 hr, and 1.0 - 5.0 µg mL^{-1}, respectively, while liquid fill volume was fixed at 1000 µL (Table 4.3). Table 4.4 shows the experimental plan for this experiment, in which the two factors were investigated in 14 runs, with six replicated centre point experiments included for estimation of pure error (see Section 4.2.1.3).

Experimental runs were carried out in triplicate (simultaneously as a single batch) using the microwell platform as described in Section 2.4.1.2, except that the values for post-induction period and concentration of dox were as detailed in Table 4.4, and all other operating conditions were fixed as detailed in Table 4.3. The same responses were measured as during the first CCF experiment (Section 2.4.2). ProSavin® titre data was entered into Design Expert software and the behaviour of the system was modelled using a quadratic fit equation. Model terms that were not significant at the level $p < 0.1$ were removed via backward elimination, starting with the least significant model terms. For the purpose of monitoring natural process variability only, ProSavin® was produced using reference shake flask cultures (as outlined in Section 2.4.1.3) parallel to this experiment.

2.4.4 Central composite circumscribed (CCC) experiment to identify the optimal settings for post-induction period and liquid fill volume

A central composite circumscribed (CCC) experiment was conducted to predict optimum factor settings (Section 4.2.1.4). Post-induction period and liquid fill volume were varied over the ranges 22 - 46 hr, and 700 - 1300 µL, respectively, while dox concentration was fixed at 3.0 µg mL^{-1} (Table 4.5). Table 4.6 shows the experimental plan for the CCC experiment, where two factors were investigated in 14 runs, and six replicated centre point experiments were included for estimation of pure error (see Section 4.2.1.4).

Experimental runs were carried out in triplicate (simultaneously as a single batch) using the microwell platform as described in Section 2.4.1.2, except that the values for post-induction period and liquid fill volume were as detailed in Table 4.6, and all other operating conditions

were fixed as detailed in Table 4.5. The same responses were measured as during the previous CCF experiments (Sections 2.4.2 and 2.4.3), plus culture pH and metabolite concentrations (glucose, lactate, glutamine and ammonium) were also quantified (methodology as described in Sections 2.11.1.3 and 2.11.1.4). ProSavin® titre and P:I ratio data was entered into Design Expert software and the behaviour of each was modelled using a quadratic fit equation. An inverse transformation was applied to titre values, in order to render the distribution approximately normal; P:I ratio data was not transformed. No terms were excluded from the model for titre, as it was judged that this yielded a model which better fitted the actual data points when viewed as a response surface plot. For the model generated using P:I ratio data, terms that were not significant at the level $p < 0.1$ were removed via backward elimination, starting with the least significant model terms. ProSavin® was produced using reference shake flask cultures (as outlined in Section 2.4.1.3) parallel to this experiment.

2.4.5 Verification of the predicted optimal settings for post-induction period and liquid fill volume based on the model fitted to the CCC experiment data

Using Design Expert software, a regression model generated from the CCC experiment data was utilised to predict optimal factor settings for maximising ProSavin® titre (Section 4.2.1.5). These were a post-induction period of ≈ 39.7 hr and a liquid fill volume of ≈ 854 µL, where the dox concentration was fixed at 3.0 µg mL^{-1}. A verification experiment was thus performed at these settings (n = 9). Harvests were also performed either side of the supposed optimum harvest time, at 15, 20, 45 and 62 hr post-induction (n = 3) to further delineate the critical impact harvest timing has on ProSavin® production. The experiment was carried out using the microwell platform as described in Section 2.4.1.2, except that the values for post-induction period and liquid fill volume were as described above, and all other operating conditions were fixed as for the CCC experiment (Section 2.4.4). The same responses were measured as during the CCF experiments (Sections 2.4.2 and 2.4.3), and end-of-culture photographs were also taken to illustrate the degree of cell aggregation using a FinePix F10 digital camera. Culture pH (Section 2.11.1.3) and osmolality (Section 2.11.1.2) were also monitored. To quantify the rate of fluid loss from microwells as a consequence of evaporation, additional microwell plates (n = 3) were seeded and induced in parallel for gravimetric analysis. The plates were weighed before seeding (to establish the weight of the vessel) and at 0 hr

(immediately post-seeding). The plates were subsequently re-weighed after 23 hr (before the addition of inducer compounds), after 25 hr (after the addition of inducer compounds), and after 38, 44, 63, 68, 85 and 92 hr. The weight of the vessel was subtracted from each measurement, which was then expressed as a percentage of the starting culture weight (the 0 hr measurement). Individual data points were plotted and the average rate of fluid loss was calculated from linear regression analysis of the 25 - 92 hr data.

2.5 Calculation of dimensionless numbers to describe the liquid phase hydrodynamics in microwells

Dimensionless numbers were used to make predictions about the fluid hydrodynamics in the shaken microwell system, based on the typical operating conditions for PS46.2 cell culture that had been established during the screening and optimisation experiments (Section 4.2.2). Reynolds number (*Re*), Froude number (*Fr*) and Phase number (*Ph*) were calculated using equations (1), (2) and (3), respectively, as described previously (Büchs *et al.*, 2000b; Büchs *et al.*, 2001b). All calculations assumed a microwell diameter of 15.6 mm, a platform orbital shaking diameter of 20 mm, a platform shaking speed of 160 rpm, and the fluid properties of water at 37 °C (ρ = liquid density, kg m^{-3}; N = shaking frequency, s^{-1}; d_f = vessel diameter, m; μ = viscosity of fluid, Pa s; d_s = shaking diameter, m; g = gravitational acceleration, m s^{-2}; V_L = liquid fill volume, m^3).

$$Re = \frac{\rho N d_f^{\,2}}{\mu} \tag{2.1}$$

$$Fr = \frac{d_s (2\pi N)^2}{2g} \tag{2.2}$$

$$Ph = \frac{d_s}{d_f}\left\{1 + 3\log_{10}\left[\frac{\rho(2\pi N) d_f^{\,2}}{4\mu}\left(1 - \sqrt{1 - \frac{4}{\pi}\left(\frac{V_L^{\frac{1}{3}}}{d_f}\right)^2}\right)^2\right]\right\} \tag{2.3}$$

2.6 Evaluation of the effect of daily dox additions on ProSavin® titre

An additional microwell experiment was conducted to further clarify the effect of dox concentration on PS46.2 productivity during extended cultures (Section 4.2.3). The addition of dox at a concentration of 1.0 µg mL^{-1} or 3.0 µg mL^{-1} (added once) was compared to a concentration of 1.0 µg mL^{-1} added every 24 hr, the purpose being to determine whether dox could have been being lost from the system (e.g. via degradation) over time, and thus full induction of cells was not being maintained over extended culture periods. As only one factor was under investigation, DoE techniques were not employed for the design of this study. ProSavin® production was carried out using the microwell platform as described in Section 2.4.1.2, except that dox concentrations were as described above, harvests were performed at 22, 40, 46 and 70 hr post-(first)induction, and all other operating conditions were fixed as for the model verification experiment (Section 2.4.5). Cell growth was monitored (Section 2.11.1.1) and ProSavin® titre was quantified according to Section 2.11.2.1 (n = 4).

2.7 Production of ProSavin® using a 2 L disposable wave-mixed bioreactor system

2.7.1 Description of the WAVE bioreactor system

A WAVE bioreactor 20/50 (GE Healthcare, Buckinghamshire, UK) was used for scale-up studies (Chapter 5). Cells were cultured within a disposable bag chamber (Cellbag; GE Healthcare), while oxygen transfer and fluid mixing were facilitated by the mechanically driven rocking motion of the bioreactor culture platform. The bioreactor base unit was used to regulate the temperature, rocking rate and rocking angle, while a separate control unit (WAVEPOD; GE Healthcare) was used to pump air mixed with CO_2 into the Cellbag. For all experiments a 2 L Cellbag with an Oxywell port was used; a probe can be inserted into the Oxywell for measurement of dissolved oxygen (see Section 2.11.1.5). The Cellbag has a fill port for large additions of inoculum or media, which is comprised of a 39” length of C-Flex® tubing that terminates in a female luer connector. A self-sealing sampling port facilitates the addition and removal of small volumes of liquid without the requirement for a tissue culture hood. Air is passed into and out of the Cellbag through 0.2 µm filters; a heater prevents the exhaust filter

from becoming blocked with condensation and a pressure release valve ensures the Cellbag remains inflated.

2.7.2 Base protocol for ProSavin® production using the WAVE bioreactor system

Here, the initial protocol used for operation of the WAVE bioreactor (Section 5.2.1.1) is detailed. Subsequent modifications to the protocol (relating to the studies described in Sections 5.2.1.2 and 5.2.2) are described in Section 2.7.3.

2.7.2.1 Inoculum preparation

Suspension-adapted PS46.2 were first expanded in shake flasks (Section 2.1.6). Cultures were pooled, then aliquoted into 50 mL centrifuge tubes and centrifuged for 5 min at 150 x *g*. Spent media was removed and cells were resuspended at a density of 1.2 x 10^6 viable cells mL^{-1} in fresh medium (containing 5 % tet-free FCS) ready to seed the Cellbag.

2.7.2.2 WAVE bioreactor operation

A liquid fill volume of 50 % (1 L) was chosen as this enabled the full capacity of the system to be exploited as stated by the manufacturer. 1 L of inoculum was syringed into a Cellbag via the fill port. The Cellbag had been pre-inflated with air comprising 5 % CO_2 and was clamped to the rocking platform; the end of the tubing used for addition of the inoculum had been retained inside a tissue culture hood to preserve sterility until addition of inoculum had been completed. The bioreactor base unit was used to set a temperature of 36.5 °C, rocking rate of 10 rocks min^{-1} and rocking angle of 6°, while the WAVEPOD pumped air mixed with CO_2 into the Cellbag at a rate of 0.1 L min^{-1}. The CO_2 concentration was set to 5 % for the duration of the culture. Induction was performed after 24 hr by addition of 3.0 µg mL^{-1} dox and 2 mM NaBu, and harvests were performed at five time points between 17 and 66 hr post-induction. Samples for measurement of cell culture parameters (cell growth [Section 2.11.1.1] and pH [Section 2.11.1.3]) were taken throughout the culture. To enable representative sampling the platform rocking rate was increased to 40 rocks min^{-1} for roughly 1 min and approximately 1.5 mL culture was removed. For vector harvests, approximately 8 mL of culture was transferred into a 15 mL centrifuge tube (BD Falcon™ conical tube; BD Biosciences) and centrifuged for 5 min at 2050 x *g*, after which the supernatant was sterile filtered using a syringe filter with 0.45 µm pore size,

and the resultant filtrate was stored in freezing vials at -80 °C. For comparison, triplicate microwell cultures were seeded, induced and harvested in parallel, using the operating conditions described in Section 2.4.5. ProSavin® titre was quantified according to the methods outlined in Section 2.11.2.1.

2.7.3 Variations in the base protocol for ProSavin® production using the WAVE bioreactor system

2.7.3.1 General modifications

For the studies described in Sections 5.2.1.2 and 5.2.2, some modifications were made to the base protocol for operation of the WAVE bioreactor. Firstly, the working volume of the Cellbag was reduced to 0.5 L, which is 25 % of the total volume. Secondly, the CO_2 concentration was reduced from 5 % to 1 % after 24 hr culture (at the time of induction), in an attempt to retain the culture pH at approximately pH 7. Thirdly, all media and inoculum additions (with the exception of when pre-coating the Cellbag – see Section 2.7.3.2) were made to the Cellbag using a sterile tubing welder. Specifically, a BIOEAZE® lid (Sigma-Aldrich) was transferred to a Nalgene® media bottle (Thermo Fisher Scientific, Wilmington, DE, USA) containing the media/inoculum inside a tissue culture hood. The C-Flex® tubing of the BIOEAZE® lid was aseptically welded to the C-Flex® tubing of the Cellbag fill port using a SCD® IIB sterile tubing welder (Terumo, Lakewood, CO, USA).

2.7.3.2 Study to determine the impact of pre-coating the Cellbag on ProSavin® titre

For the study described in Section 5.2.1.2, the impact of pre-coating the Cellbag was explored. The manufacturer recommends pre-coating the Cellbag when chemically-defined lipid supplements are used, as these can be stripped from the culture medium via an interaction with the internal surface of the culture chamber (General Electric Company, 2008b). To pre-coat, the Cellbag was first inflated with air mixed with 5 % CO_2 and clamped to the rocking platform. 0.5 L of culture medium containing 5 % (v/v) tet-free FCS was added to the Cellbag (using a syringe as described in Section 2.7.2.2) then air inlet and outlet vents were closed. The Cellbag was heated to 36.5 °C and highly agitated, using a rate of 40 rocks min^{-1} (angle 6°), so that the entire surface area of the chamber likely to be immersed during cell culture was coated. After 3 days

the medium was removed. To remove medium, a sterile tubing welder was used, as described in Section 2.7.3.1. The Cellbag was then seeded in parallel with a Cellbag that had not been pre-coated. Inoculum for each Cellbag was broadly prepared as described earlier (Section 2.7.2.1), except that cells were resuspended at a density of 1.5 x 10^6 viable cells mL^{-1} in 400 mL medium. After the cell suspension had been added to each Cellbag, a further 100 mL medium was added to rinse through any cells remaining in the tubing. For each Cellbag, this yielded a final cell concentration of 1.2 x 10^6 viable cells mL^{-1} and a final working volume of 0.5 L. Both Cellbags were then operated in parallel according to the procedures described in Section 2.7.2, excepting the modifications noted in Section 2.7.3.1. To supplement ProSavin® titre measurements, RNA copy numbers (Section 2.11.3.1) were also quantified using supernatants from the Cellbag that was *not* pre-coated.

2.7.3.3 Investigation into the effect of rocking rate on the production of ProSavin®

For the work described in Section 5.2.2, eight separate bioreactor runs were performed, using rocking rates of 6, 12, 18 and 24 rocks min^{-1} (n = 2). Inoculum was broadly prepared as described earlier (Section 2.7.2.1), with the exception that cells were resuspended at a density of 3.0 x 10^6 viable cells mL^{-1} in 200 mL medium. Inoculum was added to a Cellbag already containing 200 mL medium (used for calibration of the oxygen probe; see Section 2.11.1.5). A further 100 mL medium was then added to rinse any cells remaining in the tubing into the Cellbag. This yielded a final cell concentration of 1.2 x 10^6 viable cells mL^{-1} and a final working volume of 0.5 L. Cellbags were then operated according to the procedures described in Section 2.7.2.2, excepting the modifications noted in Section 2.7.3.1. In addition to ProSavin® titre measurements (Section 2.11.2.1), RNA copy number (Section 2.11.3.1) and product enhanced reverse transcriptase (PERT; Section 2.11.3.2) data was collected. To monitor natural process variability, ProSavin® was produced using reference shake flask cultures according to the protocol outlined in Section 2.4.1.3, parallel to all WAVE runs (parallel microwell cultures were not performed).

2.8 Stability of ProSavin® at 36.5 °C

2.8.1 Half-life of ProSavin® in crude supernatants at 36.5 °C

The decline of ProSavin® over time was characterised in cell culture supernatants incubated at a temperature of 36.5 °C (Section 6.2.1.1). To generate material for these studies, ProSavin® was produced using PS46.2 shake flask cultures as described in Section 2.4.1.3, and the resultant filtrate was stored in 50 mL centrifuge tubes at -80 °C until required. For commencement of the stability experiments, aliquots of crude supernatant were thawed and pooled.

In the first (of two) experiments, 854 µL volumes of crude supernatant were aliquoted into either a freezing vial, a static microwell plate, or a shaken microwell plate (orbital diameter 20 mm; shaking speed 160 rpm). Triplicate vials representing time 0 were placed immediately at -80 °C, while the remaining samples were incubated at 36.5 °C in a 5 % CO_2 humidified incubator. Subsequently at 24, 48, 72, 96 and 168 hr, triplicate samples for each condition were retrieved and transferred to -80 °C storage (supernatants from microwells were transferred to freezing vials prior to -80 °C storage). ProSavin® titre analysis was later performed as described in Section 2.11.2.1, and the half-life of functional ProSavin® was calculated from linear regression analysis of natural log transformed data. In addition, the samples that had been incubated in vials were analysed using the PERT assay (Section 2.11.3.2), and the half-life of viral RT was calculated from linear regression analysis of natural log transformed data (note that these samples had previously undergone two freeze-thaw cycles).

In the second experiment, 850 µL aliquots of supernatant were prepared, allowing 4 vials for titre measurements and 4 vials for RNA copy number measurements per time point. Samples representing time 0 were placed immediately at -80 °C, while the remaining vials were incubated at 36.5 °C in a 5 % CO_2 humidified incubator. Subsequently, at 3, 6, 9, 12, 24, 30, 36 and 48 hr vials were retrieved and stored at -80 °C. The rapid ProSavin® titre assay (Section 2.11.2.1) and RNA copy number assay (Section 2.11.3.1) were later performed, and the half-life of functional ProSavin® and viral RNA was calculated from linear regression analysis of natural log transformed data. In addition, the stability of viral RT was assessed using the PERT assay

(Section 2.11.3.2) (n = 4). The vials used for PERT analysis had undergone one freeze-thaw cycle, as they had formerly been defrosted for RNA copy number analysis.

2.8.2 Studies to determine if lentiviral vector losses differ in the presence or absence of producer cells

To study whether lentiviral vector losses differed in the presence or absence of producer cells (Section 6.2.1.2), an EIAV lentiviral vector that expressed the *LacZ* marker gene, and thus could be quantified in isolation from produced ProSavin® particles, was used. An EIAV lentiviral vector expressing *LacZ* that was pseudotyped with VSV-G and was, except for the difference in transgene, analogous to ProSavin® was kindly provided by the Research Group at Oxford BioMedica. This vector had been generated by transient transfection of adherent HEK293T cells, and was provided frozen (-80 °C) in crude supernatant at a reported concentration of around 3 x 10^6 TU mL^{-1}.

Two experiments were conducted. In the first experiment, the impact of cells and induction compounds on the proportion of *LacZ* vector remaining after 48 hr incubation was evaluated in four conditions (+/- cells; +/- induction reagents), as outlined in Table 2.1. Microwells were employed for this study and were kept on a shaking platform (orbital diameter 20 mm; shaking speed 160 rpm) in a 5 % CO_2 humidified incubator at 36.5 °C. Microwells were seeded using 850 µL cell suspension or fresh medium without cells, according to Table 2.1. To generate seeding material (conditions 3 and 4 only; Table 2.1), suspension cultures of PS46.2 were centrifuged for 5 min at 150 x *g*, after which the supernatant was removed and the cells were resuspended in fresh medium at a density of 1.2 x 10^6 viable cells mL^{-1}. After 24 hr, induction reagents (3.0 µg mL^{-1} dox and 2 mM NaBu) were added to microwells (conditions 2 and 4 only; Table 2.1). At this stage *LacZ* vector was also added. To achieve a final concentration of around 6 x 10^4 TU mL^{-1}, which is representative of typical concentrations of functional ProSavin® during a standard production run, 17 µL of the solution containing *LacZ* vector was added per well. Supernatant was harvested immediately from triplicate wells per condition, and after 48 hr the remaining wells were harvested (n = 3). Vector harvests were performed as described earlier (Section 2.4.1.2). The number of functional *LacZ* particles in collected supernatants was quantified using the procedure outlined in Section 2.11.2.2.

Table 2.1 Summary of the four conditions (+/- cells; +/- inducer compounds) for which the reduction in functional *LacZ* vector particles over time was evaluated. The culture medium in all instances was Freestyle™ 293 expression medium supplemented with 5 % (v/v) tet-free FCS. Six microwells were seeded for each condition. *LacZ* vector was added to all conditions at the time of induction (24 hr post-seeding); the proportion remaining after 48 hr culture was then assessed.

Condition #	Seeding material	Induction reagents added?
1	Medium only	No
2	Medium only	Yes
3	Cell suspension	No
4	Cell suspension	Yes

In the second experiment, the proportion of *LacZ* vector remaining after 24 hr incubation when diluted in two different media, that either had or had not been pre-conditioned, was compared (Table 2.2). Prior to use, the four media were transferred into 50 mL centrifuge tubes and were centrifuged for 5 min at 2050 x *g*, after which supernatants were sterile filtered using syringe filters of 0.45 µm pore size. The osmolality (Section 2.11.1.2) and pH (Section 2.11.1.3) of the four media was measured. 833 µL media and 17 µL LacZ vector was added to freezing vials (six vials per condition). Triplicate vials per condition were placed immediately at -80 °C, while the remaining vials were incubated at 36.5 °C for 24 hr, after which they too were removed to -80 °C. The number of functional *LacZ* vector particles was later quantified using the methods outlined in Section 2.11.2.2.

Table 2.2 Summary of the four conditions (two different media; +/- pre-conditioning) for which the reduction in functional *LacZ* vector particles over time was evaluated. Suspension medium was Freestyle™ 293 expression medium supplemented with 5 % (v/v) tet-free FCS, while adherent medium was that typically used for HEK293T and D17 cell culture (see Section 2.1.4). Conditioned suspension medium had been previously used for the culture of non-induced suspension-adapted PS46.2 for 3 days; conditioned adherent medium had previously been used for the culture of HEK293T for 3 days.

Condition #	Medium	Pre-conditioned?
1	Suspension medium	No
2	Suspension medium	Yes
3	Adherent medium	No
4	Adherent medium	Yes

2.9 Evaluation of different strategies for the mitigation of ProSavin® inactivation during upstream processing

2.9.1 Storage of generated material at 4 °C

Two studies were conducted to evaluate whether storage of ProSavin® at 4 °C could mitigate the rapid inactivation observed at 36.5 °C (Section 6.2.2.1). To generate material for these studies, ProSavin® was produced using PS46.2 shake flask cultures as described in Section 2.4.1.3, and the resultant filtrate was stored in 50 mL centrifuge tubes at -80 °C until required. For commencement of the stability experiments, aliquots of crude supernatant were thawed and pooled.

In the first experiment, 854µL crude supernatant was aliquoted into freezing vials. Triplicate vials representing time 0 were placed immediately at -80 °C, while the remaining samples were placed in a fridge with a temperature of 4.1 ± 0.5 °C (mean ± SD; temperature was monitored for the duration of the experiment using a TempTale®4 temperature monitor [Sensitech®, Nieuw-Vennep, The Netherlands]). 24, 48, 72, 96 and 168 hr later triplicate samples were retrieved and stored at -80 °C. ProSavin® titre analysis was later performed as described in Section 2.11.2.1. This study was carried out in parallel with the first 36.5 °C stability study described in Section 2.8.1.

In the second experiment, ProSavin® inactivation at 4 °C was evaluated over a longer time course. 850 µL crude supernatant was aliquoted into freezing vials. Four vials representing time 0 were placed immediately at -80 °C and the remaining samples were placed in a fridge with temperature 4.2 ± 0.5 °C (mean ± SD; again, temperature was monitored for the duration of the experiment using a TempTale®4 monitor). Subsequently, at 24, 48, 72, 96, 120, 144, 192, 240, 312 and 384 hr time points, quadruplet samples for each assay were retrieved and stored at -80 °C. ProSavin® titre analysis was later performed as described in Section 2.11.2.1, and RNA copy number measurements were also carried out (Section 2.11.3.1). This study was set-up in parallel with the second 36.5 °C stability study described in Section 2.8.1.

2.9.2 Reduction of production temperature to 31.5 °C

A study was conducted to evaluate whether lowering the temperature by 5 °C (to 31.5 °C) from typical production temperatures could improve titres for our system (Section 6.2.2.2). ProSavin® was produced using PS46.2 shake flask cultures broadly according to the procedure outlined in Section 2.4.1.3, except that flasks were incubated either at 31.5 or 36.5 °C (n = 4). In addition, to ensure that cells were of a similar density at the time of induction, flasks were seeded at a higher than normal density (1.6 x 10^6 viable cells mL^{-1}) and were induced much earlier (2 hr post-seeding). Vector harvests were performed 24, 44 and 67 hr post-induction, for which approximately 4 mL culture was removed (cell counts [Section 2.11.1.1] were also performed using a small proportion of this material). ProSavin® titre analysis was carried out using the methods described in Section 2.11.2.1.

2.9.3 Adjustment of cell culture osmolality by supplementation of glucose, fructose and/or sorbitol

The effect of increased osmolality (attained by supplementation of the medium with glucose, fructose and/or sorbitol) on ProSavin® titre was evaluated using microwell suspension cultures of PS46.2 (Section 6.2.2.3). A full factorial design was created using Design Expert software, whereby the concentration of each sugar type was varied over two levels (high and low). Table 6.4 shows the experimental plan in which three factors were evaluated in 12 experimental runs, with four replicated centre point experiments included for estimation of pure error.

Cell culture media supplemented with glucose, fructose or sorbitol (singularly or in combination according to Table 6.4; all from Sigma-Aldrich) were prepared as described here. Fructose and sorbitol were provided as dry powders and thus required dissolving in tissue culture grade water (Sigma-Aldrich) prior to use, while glucose was supplied as a ready-to-use 45 % (w/v) sterile solution. For each experimental condition, a concentrated solution of glucose, fructose, and/or sorbitol was prepared and sterile filtered through a 0.2 µm filter (Sartorius). The concentrated solution was then added to cell culture medium, such that the solution comprised 9.3 % (v/v) of the final volume, and the resulting concentration of glucose, fructose, and/or sorbitol was as stated in Table 6.4. For one experimental condition, neither glucose, fructose nor sorbitol were required; in this instance cell culture medium containing 9.3 % (v/v) tissue culture grade water

was used. The osmolality of the different media was measured (as was the osmolality of standard culture medium *without* supplementation or partial dilution with water).

Suspension-adapted PS46.2 were initially transferred into shake flasks containing fresh media supplemented with glucose, fructose and/or sorbitol at the concentrations stated in Table 6.4, and cultured for 3 days using standard conditions as described in Section 2.1.6. To initiate the microwell experiment, cultures were then centrifuged for 5 min at 150 x *g*, supernatant was removed and cells were resuspended at a density of 2 x 10^6 viable cells mL^{-1} in fresh medium (supplemented according to Table 6.4). Experimental runs (Table 6.4; including, for comparison, cells in standard non-modified medium) were seeded in triplicate using the microwell platform, with 850 µL culture per well. Microwells were kept on shaking platform (orbital diameter 20 mm; shaking speed 160 rpm) in a 5 % CO_2 humidified incubator at 36.5 °C. To ensure that cells were of a similar density at the time of induction, induction reagents (3.0 µg mL^{-1} dox and 2 mM NaBu) were added to microwells 2 hr post-seeding (note that the seeding density was higher than that typically used, to compensate for this short post-induction period). Cell density measurements (Section 2.11.1.1) and vector harvests were carried out 23, 44 and 65 hr post-induction (n = 1 at each time point). Vector harvests were carried out as described earlier (Section 2.4.1.2). Titres were quantified using the rapid ProSavin® titre assay (Section 2.11.2.1).

Parallel to this experiment, ProSavin® was produced in shake flasks according to the protocol outlined in Section 2.4.1.3. The generated material was utilised to assess whether the presence of different sugar types in the culture medium impacted on the transduction stage of the titre assay and thus indirectly influenced final titre measurements. Immediately prior to cell transductions, supernatants harvested from shake flask cultures were thus split into several aliquots, which were supplemented such that the resulting concentrations of the different sugar types were as stated in Table 6.4. Titres were otherwise determined as normal (Section 2.11.2.1).

2.10 Comparison of the stability of different lentiviral vector preparations incubated at 36.5 °C

2.10.1 Description of experiments

Experiments were conducted to examine whether the choice of production system (transient transfection *versus* stable producer cell lines) and vector genome (ProSavin®, Vector 13.1 or enhanced green fluorescent protein [eGFP]) influenced the stability of generated lentiviral particles (Section 6.2.3). ProSavin® was generated using adherent PS46.2 cultures as described in Section 2.10.2, while ProSavin®, Vector 13.1 (a lentiviral vector that expresses a candidate therapeutic transgene) and eGFP lentiviral vectors were produced via transient transfection as outlined in Section 2.10.3. Aliquots of crude supernatant were thawed and small volumes of 70, 320 and 1300 µL (for later analysis using the PERT, RNA copy number and titre assays, respectively) were dispensed into freezing vials. Triplicate vials of each volume were prepared for each time point to be assessed (7 time points in total). The time points were chosen based on preliminary studies (data not shown), such that the depletion of functional particles to low levels (but still within the quantifiable range of titre assays) could be observed. Vials representing time 0 were placed immediately at -80 °C, while the remaining samples were incubated at 36.5 °C in a 5 % CO_2 humidified incubator. Vials of ProSavin® that had been generated using adherent PS46.2 cultures were subsequently moved to -80 °C storage after 8, 24, 32, 48, 72 and 96 hr incubation. Vials containing supernatant that had been produced by transient transfection of adherent HEK293T were moved to -80 °C storage after incubation for 2, 4, 6, 8, 12 and 24 hr (ProSavin®), 8, 24, 32, 48, 72 and 96 hr (Vector 13.1), and 8, 24, 48, 72, 144 and 240 hr (eGFP vector). ProSavin® titres were quantified using two methods (Sections 2.11.2.1 and 2.11.2.3); however, one set of cell transductions was performed and the cells were split 3 days later so that each assay could be continued in isolation. Vector 13.1 and eGFP vector titres were quantified using the DNA integration assay (Section 2.11.2.3) only. RNA copy number (Section 2.11.3.1) and PERT (Section 2.11.3.2) analysis was carried out for all preparations. The half-life of functional vector and viral RNA was calculated from linear regression analyses of natural log transformed data.

A second batch of ProSavin® was produced by transient transfection of HEK293T, and also tested (n = 1). Crude vector was aliquoted into freezing vials (650 µL per vial); vials were moved to -80 °C storage after 0, 4, 8, 22 and 32 hr incubation. Biological titre was quantified using the rapid ProSavin® titre assay (Section 2.11.2.1).

2.10.2 Production of ProSavin® using adherent stable producer (PS46.2) cells

ProSavin® for use in stability studies was produced using adherent cultures of PS46.2. Adherent PS46.2 were seeded in 10 cm dishes (BD Falcon™ tissue culture treated; BD Biosciences) at a density of 1 x 10^7 viable cells mL^{-1} in 10 mL medium. Cells were kept in a 5 % CO_2 humidified incubator at 36.5 °C, and were induced after 20 - 22 hr by addition of 1.0 µg mL^{-1} dox and 10 mM NaBu. Medium was replaced 6 - 8 hr post-induction with 6 mL medium containing 1.0 µg mL^{-1} dox (without NaBu). 20 - 23 hr later harvests were performed: supernatants were sterile filtered using syringe filters of 0.45 µm pore size and the resultant filtrate was stored in 15 mL centrifuge tubes at -80 °C.

2.10.3 Production of lentiviral vectors by transient transfection of adherent HEK293T cells

Vectors for use in stability studies were also produced by three plasmid transient co-transfection of adherent HEK293T. The codon-optimised EIAV Gag/Pol expression plasmid pESGPK (Stewart *et al.*, 2009) and VSV-G envelope expression plasmid pHGK were used in all instances (Figure 2.1). pHGK is a derivative of phG[VSVG2] (Farley *et al.*, 2007) in which the ampicillin selectable marker has been replaced with a kanamycin resistance gene. For production of ProSavin®, the genome expression plasmid pONYK1 was used. The vector construct expressed by pONYK1 is identical to that previously described for pONYK1-ORT (Farley *et al.*, 2007; Jarraya *et al.*, 2009; Stewart *et al.*, 2009; Stewart *et al.*, 2011); pONYK1 differs from pONYK1-ORT only in that it contains a kanamycin selectable marker on the plasmid backbone, i.e outside the LTRs. Vector 13.1 was generated using the genome plasmid pONYK13.1 which encodes a candidate therapeutic gene, and eGFP vectors were produced using the genome plasmid PONYKG, which encodes the *eGFP* reporter gene. All vector constructs are displayed in Figure 2.1. Plasmids were kindly provided by the Research Group at Oxford BioMedica.

For transient transfections, 10 cm dishes were seeded with HEK293T cells at a density of 3.5 x 10^5 viable cells mL^{-1}, using 10 mL medium per dish. Dishes were incubated at 36.5 °C in a 5 % CO_2 humidified incubator. Three-plasmid transient transfections were performed 21 - 26 hr later using Lipofectamine® 2000 CD transfection reagent (Invitrogen). 25 µL Lipofectamine® 2000 CD was added drop-wise to 315 µL OptiPRO™ medium (Invitrogen) and incubated at room temperature for 5 min. Meanwhile, the following were combined with 340 µL OptiPRO™: 2 µg EIAV Gag/Pol expression plasmid, 0.08 µg VSV-G envelope plasmid, and 4 µg genome plasmid. The plasmid mix was then combined with the Lipofectamine® 2000 CD mix, and the whole was incubated at room temperature for 15 - 45 min before being added to cells. Cells were induced 14 - 19 hr post-transfection with 10 mM NaBu; medium was replaced 6.5 - 8 hr later with 6 mL fresh medium (not containing NaBu). Vector harvests were performed 21 - 23 hr following medium exchange: supernatants were sterile filtered using syringe filters of 0.45 µm pore size, and the resultant filtrate was stored in 15 mL centrifuge tubes at -80 °C.

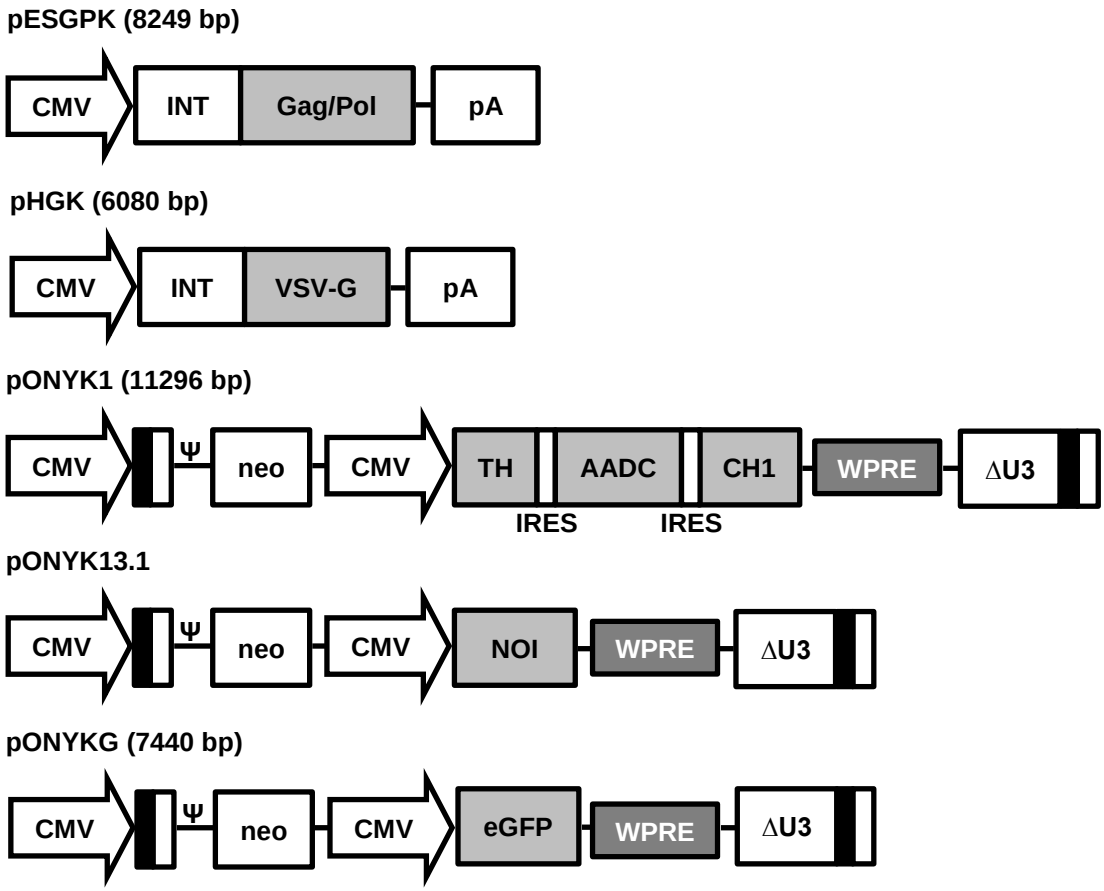


Figure 2.1 Diagram of lentiviral vector constructs. Each construct is encoded by a different plasmid, as indicated. Full-length equine infectious anaemia virus (EIAV) vector genome transcripts are expressed from an immediate-early cytomegalovirus (CMV) / EIAV long terminal repeat (LTR) RU5 chimeric promoter. All vectors contain a deletion in the U3 region (ΔU3) of the 3'-LTR causing self-inactivation (SIN) of the vector upon transduction. The ProSavin® transgenes (tyrosine hydroxylase [TH], aromatic L-amino acid decarboxylase [AADC] and GTP cyclohydrolase 1 [CH1]), Vector 13.1 transgene (Nucleotide of Interest (NOI)) and enhanced green fluorescent protein (eGFP) reporter gene are indicated where relevant. Also indicated (where present) are: Gag/Pol, vesicular stomatitis virus glycoprotein (VSV-G), intron (INT), polyadenylation signal (pA), EIAV packaging signal (Ψ), neomycin (neo) open reading frame (ORF), internal ribosome entry site (IRES), and woodchuck hepatitis virus post transcriptional regulatory element (WPRE). Plasmid size is given in base pairs (bp). All plasmids contain a kanamycin *Escherichia coli* selectable marker.

2.11 Analytics

2.11.1 Analytical techniques for the measurement of cell culture parameters

2.11.1.1 Cell density and viability

Cell number and viability were determined using a Cedex XS cell counter (Roche Diagnostics Ltd, Burgess Hill, UK) and the trypan blue exclusion method (as described by the manufacturer). The Cedex XS is a semi-automated cell counter that employs digital image recognition technology to enumerate live and dead cells. Samples diluted in trypan blue (Roche Diagnostics Ltd) are loaded onto a Cedex Smart Slide (Roche Diagnostics Ltd) and then analysed. A sample size of approximately 10 µL is required, and eight samples can be analysed using a single slide.

2.11.1.2 Osmolality

Cell culture samples of 50 µL volume were analysed using a cryoscopic OSMOMAT® 030 osmometer (Gonotec GmbH, Berlin, Germany), which uses the freezing point depression of a solution to calculate osmolality. Prior to sample measurements, a two-point calibration was performed using distilled water and a 300 mOsmol kg^{-1} calibration solution (Gonotec). This yields a measuring range of 0 - 700 mOsmol kg^{-1} with ± 1 % linearity (gonotec, 2006).

2.11.1.3 pH

For studies described in Chapters 3 and 4, cell culture pH was measured immediately post-sampling using a blood gas analyser (ABL800 BASIC; Radiometer Ltd, West Sussex, UK), which had been calibrated according to the manufacturer's instructions. This instrument requires a sample volume of 195 µL. As the blood-gas analyser was unavailable for use during the later studies described in Chapter 5, pH indicator strips (pH-Fix 6.0 - 7.7; Fisher Scientific; catalogue number FB33013) were utilised instead. The colour of the indicator strip, directly following immersion in the sample solution, was compared with a reference chart in order to determine pH.

2.11.1.4 Concentration of metabolites

A YSI 7100 multiparameter bioanalytical system (YSI Inc, Yellow Springs, OH, USA), which had been calibrated according to the manufacturer's instructions, was used to measure the concentration of glucose (D-glucose), lactate (L-lactate), glutamine (L-glutamine) and ammonium (ammonium ions, $NH4^+$). Culture samples (~ 1 mL) were transferred into 1.5 mL microcentrifuge tubes (Eppendorf UK LTD, Stevenage, UK) and stored at -20 °C until analysis, which was carried out within one month. Note that a sample volume of 20 - 25 µL is required for analysis of each metabolite. For the study described in Section 4.2.1.4, culture samples were first centrifuged (2300 x *g* for 1 min) and the supernatant was transferred into a fresh microcentrifuge tube prior to -20 °C storage. This step was introduced as a precaution to prevent the instrument becoming clogged with cell debris. The YSI determines the concentrations of glucose, lactate and glutamine (separately) using enzyme sensor technology, while ammonium concentration is determined using an ion selective electrode (see YSI Incorporated (2003) for more details).

2.11.1.5 Dissolved oxygen in WAVE bioreactor cultures

For measurement of dissolved oxygen in WAVE bioreactor cultures, a dissolved oxygen optical probe (DOOPT-PROBE; GE Healthcare) was attached to the WAVEPOD control unit. The probe contains immobilised dye molecules whose luminescence is quenched by molecular oxygen (General Electric Company, 2008c). Outside of the Cellbag, the probe was initially calibrated to 0 % and 100 % air saturation, using a zero oxygen standard solution (GE Healthcare) and gaseous air, respectively. The probe was then inserted into the Oxywell port of a Cellbag containing 200 mL culture medium. The Cellbag was rocked at a rate of 18 rocks min^{-1} using an angle of 2°, such that the probe was continually immersed in medium. The Cellbag was incubated at 36.5 °C while air comprising 5 % CO_2 was continually passed through the headspace at a rate of 0.1 L min^{-1}. After approximately 20 min, when the oxygen reading had plateaued, the 100 % calibration was re-performed (to adjust for the effects of liquid immersion and the shift in temperature on oxygen levels since the initial calibration).

2.11.2 Analytical techniques for the quantification of lentiviral vector biological titre

2.11.2.1 Rapid ProSavin® titre assay (flow cytometry)

The ProSavin® titre assay using flow cytometry was the most common method employed to quantify functional lentivirus vector titres. As compared to the DNA integration assay, high numbers of samples can be processed in a relatively short time frame. This assay uses the tyrosine hydroxylase (TH) enzyme, expressed from the ProSavin® transgene cassette, as a marker to identify cells that have been successfully transduced by ProSavin®. Target cells that had been exposed to ProSavin®-containing supernatants underwent immunostaining, to fluorescently label TH-expressing cells, and were then analysed using flow cytometry.

Target cells (adherent HEK293T) were seeded in 12-well tissue culture plates (BD Falcon™; BD Biosciences) at a density of 9 x 10^4 viable cells mL^{-1} in 1 mL medium per well. Cells were incubated at 36.5 °C in a 5 % CO_2 humidified incubator. 16 - 28 hr later, cells in at least two representative wells were counted. Provided that densities were between 1 x 10^5 and 3 x 10^5 viable cells mL^{-1}, transductions were subsequently performed. Dilutions of ProSavin® containing supernatants were prepared using standard HEK293T culture medium supplemented with polybrene (final concentration 6 - 8 µg mL^{-1}; Sigma-Aldrich). Polybrene is a polycation regularly reported to enhance retroviral vector transduction efficiency (Davis *et al.*, 2002). Similar titres were attained whether 6 or 8 µg mL^{-1} was polybrene used (data not shown). Culture medium was removed from target cells and replaced with 0.5 mL diluted vector. Wells were topped-up 3 - 6 hr later with 1 mL fresh medium. 3 days later cells were transferred to a 96-well U-bottom plate to enable immunostaining. Cells were fixed for 15 min using fixative solution (Fix & Perm Medium A; Invitrogen), then incubated for 1 hr in the presence of a rabbit anti-tyrosine hydroxylase antibody (Millipore, Hayward, CA, USA) diluted 1:500 in permeabilisation solution (Fix & Perm Medium B; Invitrogen). Cells were incubated for a further 1 hr in the presence of an Alexa Fluor® 488 goat anti-rabbit antibody (Invitrogen) diluted 1:500 with permeabilisation solution. Cells were washed with PBS prior to each step of the immunostaining procedure, and, following centrifugation at 2050 x *g* for 5 min, supernatant was discarded at each stage by rapid inversion of the plate. Following immunostaining, cells were

re-suspended in PBS and stored at 4 °C or kept on ice until analysis by flow cytometry. Negative and positive (reference) controls were included in each assay.

Flow cytometry was performed using either a BD FACSCalibur™ or BD FACSVerse™ flow cytometer (both BD Biosciences). Comparable titre results were attained when the same set of samples was analysed by both instruments (data not shown). Size and fluorescence data was collected for 10,000 events per sample. Data was transferred to WinList™ software (version 5.0 or 7.0; Verity Software House, Topsham, ME, USA) for analysis. WinList™ software was utilised to obtain the percentage of HEK293T cells that exceeded a set fluorescence threshold based on background fluorescence of non-transduced cells (Figure 2.2). Cells exceeded this threshold if they had been transduced by ProSavin®, were producing tyrosine hydroxylase, and had been labelled by the immunostaining protocol. To minimise the likelihood of analysing cells with multiple copies of vector, results were only considered valid if less than 20 % of cells exceeded the set fluorescence threshold (Sastry *et al.*, 2002). Thus, assuming one transducing unit per transduced cell, ProSavin® titre (in terms of TU mL^{-1}) could be calculated using the following equation: titre = [(% transduced cells / 100) x number of cells per well at transduction x dilution factor)] / volume of vector in mL.

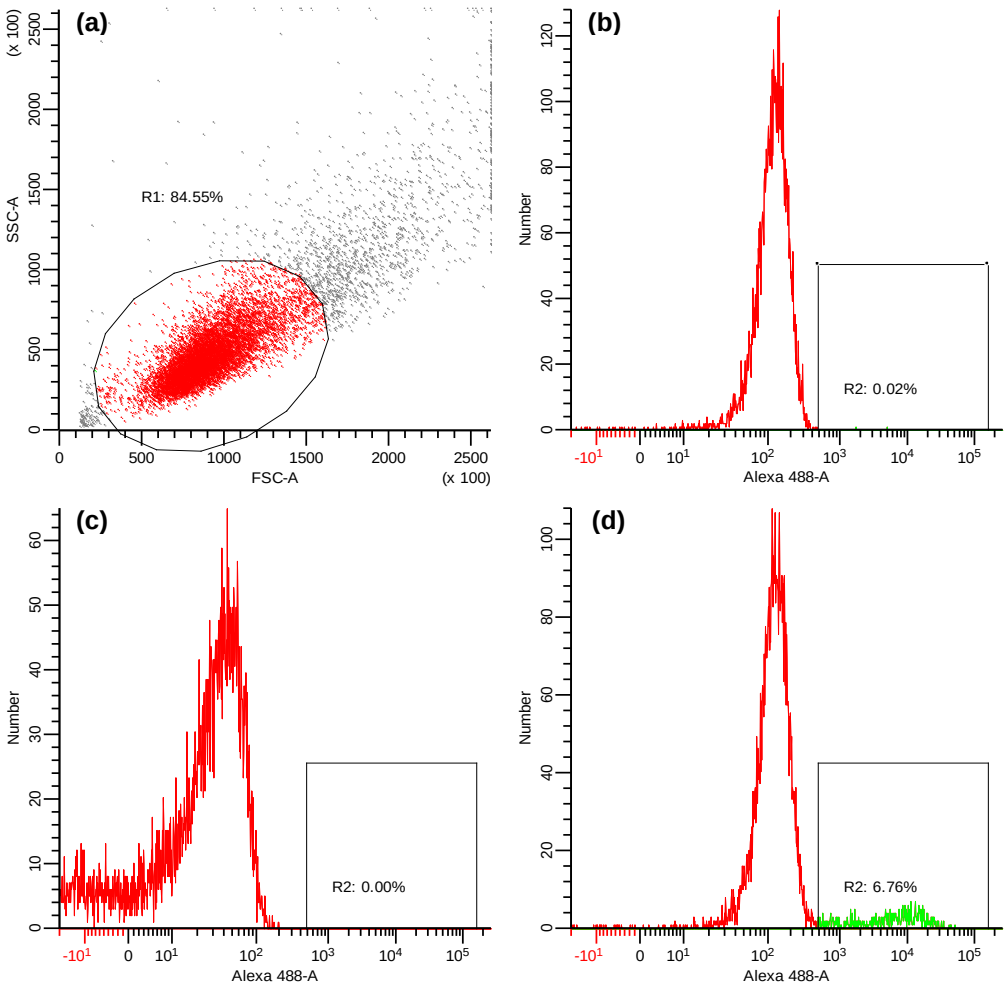


Figure 2.2 Example illustration of the method utilised to determine the proportion of positive tyrosine hydroxylase (TH)-expressing cells in a given sample following flow cytometry. Plots were generated using WinList™ software. Scatter plot (a) illustrates the forward scatter (FSC) and side scatter (SSC) of a non-transduced (negative) fluorescent-stained HEK293T population. An elliptical gate (R1) has been drawn around the cell population to exclude debris from further analyses. Only cells within the R1 region are subsequently analysed; once drawn, this region applies for all samples within a given assay. In histogram (b), Alexa 488 fluorescence of R1 cells (same population as displayed in (a)) is presented. Here, the cell population has been utilised to set a threshold above which positive TH-expressing cells may be discriminated from background fluorescence (R2). Once drawn, this region also applies for all samples within a given assay. Once R1 and R2 have been specified, analysis of test samples can proceed. For illustrative purposes, histogram (c) shows the fluorescence of a non-transduced cell population that has *not* been stained with fluorescent antibodies. Mean fluorescence is greatly reduced compared to that observed in histogram (b), demonstrating the importance of using *stained* non-transduced cells for defining R2. Histogram (d) shows the fluorescence of cells transduced with ProSavin® that was generated from standard shake flask cultures (crude supernatants were diluted 1 in 2). Cells within the R2 region are expressing TH; the percentage denoted is utilised to calculate titre. A linear relationship exists between dilution factor and the observed percentage of positive (TH-expressing) cells (data not shown).

2.11.2.2 Rapid titre assay for lentiviral vectors expressing *LacZ*

To quantify vectors containing the *LacZ* marker gene, transduced target cells were stained with X-Gal. Cell colonies expressing β-galactosidase (the enzyme encoded by the *LacZ* gene) could then be visualised using an optical microscope and enumerated. Target cells (adherent D17) were seeded in 12-well tissue culture plates at a density of 8 x 10^4 viable cells mL^{-1} in 1 mL medium per well. Cells were incubated at 36.5 °C in a 5 % CO_2 humidified incubator. 16 - 28 hr later, duplicate dilution series were prepared for each vector sample using standard D17 culture medium supplemented with polybrene (final concentration 8 µg mL^{-1}). Culture medium was removed from target cells and replaced with 0.5 mL diluted vector. Positive and negative (untransduced) controls were also included in each assay. 3 - 6 hr later wells were topped up with 1 mL medium. 3 days later culture medium was removed and cells were fixed for 5 min using 0.5 mL formalin (VWR, Lutterworth, UK). Cells were washed with PBS and 0.5 mL X-Gal stain was added. X-Gal stain consisted of 0.4 mg mL^{-1} X-Gal (Melford Laboratories, Ipswich, UK; from a 20 mg mL^{-1} stock that had been prepared using N, N-dimethylformamide [Sigma-Aldrich]), 2 mM $MgCl_2$ (Sigma-Aldrich), 4 mM potassium ferrocyanide (Sigma-Aldrich), and 4 mM potassium ferricyanide (Sigma-Aldrich), in PBS. X-Gal, $MgCl_2$, potassium ferrocyanide and potassium ferricyanide were kindly supplied by the Research Group at Oxford BioMedica. Cells were incubated at 36.5 °C. The next day, blue β-galactosidase-expressing colonies were visualised using an optical microscope and, for wells containing 20 - 200 colonies, the number of colonies per well was counted. Titre (in terms of TU mL^{-1}) was then calculated using the following equation: titre = (number of colonies per well x dilution factor) / volume at transduction in mL.

2.11.2.3 DNA integration assay

The DNA integration assay has been previously described in detail (Martin-Rendon *et al.*, 2002; Radcliffe *et al.*, 2008; Stewart *et al.*, 2009). Briefly, this assay uses quantitative real-time PCR (q-PCR) and primers specific to the EIAV packaging signal (Ψ) to quantify the amount of proviral DNA in transduced target cells. Target cell seeding and vector transductions were carried out as described earlier (Section 2.11.2.1). Transduced cells were passaged every 2 - 3 days for 10 days to eliminate non-integrated vector DNA. Cells were harvested by transferring

approximately 1.5 x 10^6 viable cells mL^{-1} into a 1.5 mL microcentrifuge tube, which was centrifuged at 300 x *g* for 5 min. Supernatant was removed and cell pellets were stored at -80 °C. DNA was extracted from cell pellets using a QIAamp DNA Blood Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. DNA was resuspended in a final volume of 80 µL and stored at -20 °C. For DNA quantitation based on absorbance measurement at 260 nm, a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) was utilised according to the manufacturer's instructions. DNA results generated using the NanoDrop 1000 matched those attained when the same samples were analysed using a Beckman Du® 640 spectrophotometer (Beckman Coulter, High Wycombe, UK; data not shown).

To set up TaqMan® reactions a PCR mix was prepared, which contained 15 µL TaqMan® Universal PCR Master Mix (Applied Biosystems®; Invitrogen), 300 nM EIAV Ψ forward primer (5' ATT GGG AGA CCC TTT GAC ATT G 3'), 300 nM Ψ reverse primer (5' ACC AGT AGT TAA TTT CTG AGA CCC TTG TA 3') and 200 nM EIAV Ψ probe (5' [FAM] CAC CTT CTC TAA CTT CTT GAG CGC CTT GCT [TAMRA] 3'), made up to a total volume of 24 µL. The concentrations of primers and probe are provided in terms of the final volume (30 µL), which is reached by the addition of 6 µL DNA. Each test sample was analysed in duplicate using 50 - 750 ng DNA per reaction. A plasmid DNA standard containing EIAV Ψ of known copy number was used to generate a dilution series for a standard curve. In addition, DNA derived from a transduced clonal cell line containing one copy of EIAV vector genome per cell (the 'single integrant' or 'SI') was quantified in parallel to test samples. The primers, probe, DNA standard and SI were obtained from the PAR Group at Oxford BioMedica. Appropriate negative and positive (reference) controls were also included in each assay. Following the addition of DNA to the PCR mix, 25 µL was transferred to a 96-well optical plate (Applied Biosystems®) and the number of copies of Ψ per reaction was quantified using an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems®). Cycling conditions were: 50 °C for 2 min; 95 °C for 10 min; 95 °C for 15 sec; 60 °C for 1 min. The last two steps were repeated for 40 cycles.

Data analysis was performed using the supported Sequence Detection System software package according to the manufacturer's recommendations. The cycle threshold (Ct) value is the cycle at which the fluorescent signal of a given reaction significantly exceeds the background level; this parameter thus reflects the amount of DNA template. Data was

scrutinised to ensure the following criteria were satisfied: (i) duplicate reactions returned Ct values within one cycle of each other, (ii) at least three data points were used to construct the standard curve, (iii) the R^2 of the trendline fitted to standard data was ≥ 0.98, (iv) the reaction efficiency (calculated from the slope of the trendline) was between 83 and 115 %, (v) all sample data were within the quantifiable range of the assay and finally (vi) significant fluorescent signal was detected in the positive control (reference) reactions, and not detected in negative control reactions.

To determine titres from Ψ data, values were first adjusted to account for the amount of input DNA. The number of Ψ copies *per cell* was then determined by dividing the number of copies of Ψ in unknown (test) samples by the number of copies of Ψ in SI DNA. Titre (in terms of TU mL^{-1}) could then be calculated using the following equation: titre = [(number of copies of Ψ per cell x number of cells per well at transduction x dilution factor)] / volume of vector in mL.

2.11.3 Analytical techniques for the quantification of total lentiviral vector particle numbers

2.11.3.1 RNA copy number assay

The RNA copy number assay was performed similarly to as previously described (Martin-Rendon *et al.*, 2002; Rohll *et al.*, 2002; Stewart *et al.*, 2009). In brief, this assay uses quantitative real-time RT PCR (qRT-PCR) and primers specific to the EIAV Ψ to quantify the number of genomic RNA molecules in a vector preparation. RNA was isolated from 140 µL crude supernatant (or 25 µL crude supernatant diluted in 115 µL PBS) using a QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was resuspended in a final volume of 60 µL. Removal of residual DNA was performed using a DNA-*free*™ Kit (Ambion®; Invitrogen). 5 µL extracted RNA was combined with 3 µL DNaseI (2 U $µL^{-1}$), 5 µL incubation buffer and 37 µL water and incubated at 37 ± 1 °C for 1 hr. The reaction was stopped by incubation at room temperature for 2 min in the presence of 10 µL inactivation reagent. The inactivation reagent was subsequently pelleted by centrifugation and the RNA-containing supernatant transferred to a fresh tube.

To set up TaqMan® reactions a PCR mix was prepared, which contained 15 µL TaqMan® One-Step RT-PCR Master Mix, 0.75 µL MultiScribe™ RT and RNase Inhibitor Mix (Applied Biosystems®), 300 nM EIAV Ψ forward primer, 300 nM Ψ reverse primer and 100 nM EIAV Ψ probe, made up to a total volume of 27 µL. The concentrations of primers and probe are provided in terms of the final volume (30 µL), which is reached by the addition of 3 µL RNA template. The nucleotide sequences of the primers and probe are as stated in Section 2.11.2.3. A second PCR mix was prepared in which the MultiScribe™ RT and RNase Inhibitor Mix was omitted, in order to assess DNA contamination levels. Each RNA template was analysed in duplicate (+RT) and singularly (-RT). 'Unknown' samples were diluted 10^0 - 10^2. A cRNA standard containing EIAV Ψ of known copy number was used to generate a dilution series for a standard curve. The primers, probe and cRNA standard were obtained from the PAR Group at Oxford BioMedica. Appropriate negative and positive (reference) controls were also included in each assay. Following addition of template RNA to PCR mix, 25 µL was transferred to a 96-well optical plate and RNA copies mL^{-1} were quantified using an ABI Prism® 7900HT Sequence Detection System. Cycling conditions were: 48 °C for 30 min; 95 °C for 10 min; 95 °C for 15 sec; 60 °C for 1 min. The last two steps were repeated for 40 cycles. If RNA extractions, DNaseI treatment and set-up of the TaqMan plate could not be completed sequentially on the same day, extracted RNA was stored in the interim at either -20 °C (up to 5 days) or -80 °C (up to 6 months).

Data analysis was performed using Sequence Detection System software according to the manufacturer's recommendations, and data was scrutinised to ensure the assay acceptance criteria were met. Criteria were as outlined in Section 2.11.2.3, plus the following condition was added: (vii) the DNA copy number returned in each –RT reaction should be at least 10-fold lower than the RNA copy number returned for parallel +RT reactions.

2.11.3.2 Product enhanced reverse transcriptase (PERT) assay

The PERT assay was performed using a method similar to that previously described (Martin-Rendon *et al.*, 2002; Rohll *et al.*, 2002; Stewart *et al.*, 2009). This assay uses a mild detergent to release RT associated with lentiviral vector particles. qRT-PCR is then performed, in which the RT liberated from vector particles is utilised to convert MS2 (bacteriophage) RNA into cDNA.

The amount of cDNA synthesised is proportional to the amount of RT present in the reaction, and as the amount of RT present in the reaction directly relates to the number of lentiviral vector particles initially present within the sample, a prediction of biological titre (PERT predicted TU mL^{-1}) can accordingly be made.

To disrupt vector particles, 25 µL sample (crude supernatant) was added to 25 µL disruption buffer (40 mM Tris-HCl [pH 7.5], 50 mM KCl, 20 mM DTT, 0.2 % [v/v] Igepal® CA-630) (Martin-Rendon *et al.*, 2002; Rohll *et al.*, 2002). Serial dilutions of disrupted vector stocks were carried out using PBS (10^2 - 10^3). In parallel to unknown (test) samples, an EIAV-based lentiviral vector preparation of known biological titre was disrupted and diluted to generate a standard curve. The disruption buffer and vector for creation of the standard curve were obtained from the Research/PAR Group at Oxford BioMedica. To set up TaqMan® reactions a PCR mix was prepared that consisted of 15 µL TaqMan® Universal PCR Master Mix No AmpErase UNG (Applied Biosystems®), 0.4 U μL^{-1} RNase Inhibitor (Ambion®), 300 nM PERT forward primer (5' TTC TGC TCA ACT TCC TGT CGA 3'), 300 nM PERT reverse primer (5' CAC AGG TCA AAC CTC CTA GGA ATG 3') and 150 nM PERT probe (5' [FAM] CGA GAC GCT ACC ATG GCT ATC GCT GTA G [TAMRA] 3') made up to a total volume of 26.78 µL. The concentrations of RNase Inhibitor, primers and probe are provided in terms of the final volume (30 µL), which is reached by the addition of 0.225 µL MS2 RNA (Roche Diagnostics Ltd) and 3 µL sample (source of RT). The primers and probe were obtained from the Research/PAR Group at Oxford BioMedica, and are specific for MS2 RNA. Negative controls (-RNA template or -RT source) were also included in each assay. Once the MS2 RNA and disrupted sample and had been added to the PCR mix, 25 µL was transferred to a 96-well optical plate and PERT predicted TU mL^{-1} was quantified using an ABI Prism® 7900HT Sequence Detection System (cycling conditions as described in Section 2.11.3.1).

Data analysis was performed using Sequence Detection System software according to the manufacturer's recommendations. Data was scrutinised to ensure the assay acceptance criteria were met. Criteria were as outlined in Section 2.11.2.3, except a broader range in terms of reaction efficiency (60 - 115 %) was accepted.

2.11.4 Software

Unless otherwise stated, Excel (Microsoft®, Berkshire, UK) was used to solve all fluid mixing, lentiviral vector titre, and lentiviral vector particle number calculations. Except where stated, Origin Pro 9 software (OriginLab Corporation, Northampton, MA, USA) was used for linear regression analyses and to create all graphs.

3 Development of a microwell platform for use in lentiviral vector bioprocess development

3.1 Introduction and aims

Lentiviral vectors have the capacity to stably integrate a therapeutic gene of interest into the genome of both dividing and non-dividing cells *in vivo*, conferring long-term gene expression (Naldini *et al.*, 1996a; Naldini *et al.*, 1996b). Lentiviral vectors are thus efficient gene delivery tools, and several promising candidates are in development for a range of gene therapy applications (see Section 1.3.2). The first clinical trial to utilise a lentiviral vector was initiated in 2003 (Manilla *et al.*, 2005; Levine *et al.*, 2006) and since then (up to January 2014) 71 lentiviral vector-based clinical trials have been undertaken (http://www.wiley.co.uk/genmed/clinical/ accessed 26.02.14). While lentiviral vectors continue to make good progress in the clinic, of mounting concern is whether existing manufacturing processes are capable of generating adequate quantities of material to fulfil expected commercial demands (Ansorge *et al.*, 2010; Schweizer and Merten, 2010; Segura *et al.*, 2013). Lentiviral vectors used in clinical trials are currently produced in multi-layered cell factories by transient transfection of adherent human embryonic kidney (HEK)293 cell lines (or their derivatives) using between two and five plasmids (Slepushkin *et al.*, 2003; Negré *et al.*, 2008; Schweizer and Merten, 2010; Merten *et al.*, 2011; Stewart *et al.*, 2011; Ausubel *et al.*, 2012). This approach is not, however, suited to the generation of a licensed product. Transient transfection techniques are costly and technically challenging to scale-up and batch-to-batch variability is common, while the presence of large quantities of transfected plasmid DNA may exacerbate the risk of replication competent lentivirus (RCL) formation (Cockrell and Kafri, 2007; Stewart *et al.*, 2009; Ansorge *et al.*, 2010; Schweizer and Merten, 2010; Rodrigues *et al.*, 2011; Segura *et al.*, 2013). Adherent cell expression systems are also not amenable to scale-up and the growth of HEK293-derived cells as monolayers means that production capacity can only be increased by “scaling-out”, through introducing more cell factories (Schweizer and Merten, 2010). Consequently, for product commercialisation, the development of producer cell lines that have all genes for vector production stably integrated and are amenable to growth in suspension is highly desirable. This would eliminate the requirement for a transient transfection step and mean that scale-up into

multi-litre bioreactors would also become feasible. However, the successful creation of a suspension-grown lentiviral vector producer cell line that generates commercially-relevant titres has been reported just once (Broussau *et al.* (2008); see Section 1.4.1). This is clearly, therefore, an area warranting significant attention. To date, progress has likely been hindered by the difficulties encountered when developing suspension-adapted producer cell lines. In particular, the tendency of HEK293-derived cells to aggregate when cultured in suspension (Merten *et al.*, 2001), changes in the morphology and membrane properties of cells following suspension adaption (Ansorge *et al.*, 2010), and the use of non-optimised culture conditions (Segura *et al.*, 2013) can all play a role in diminishing titres. To aid the development of scalable manufacturing processes for lentiviral vectors, a deeper understanding of the factors impacting on titres from suspension cultures of stable producer cell lines is clearly required. As a range of parameters may influence titres, numerous experiments are needed to characterise their various effects. Microscale bioprocessing combined with Design of Experiments (DoE) techniques could simplify this process (see Sections 1.5.1 and 1.5.2, respectively). Orbitally shaken microwell plates are low-cost and simple to use, require little space and culture materials, and allow numerous parameters to be evaluated quickly and simultaneously (Kumar *et al.*, 2004; Neubauer *et al.*, 2013). As compared to the traditional one-factor-at-a-time (OFAT) approach to process optimisation, DoE better characterises the effects of multiple parameters using a smaller set of experiments that are linked in a rational manner (Czitrom, 1999; Tye, 2004; Anderson and Whitcomb, 2007; Eriksson *et al.*, 2008). The value of combining microwell experimentation and DoE techniques has been demonstrated for the optimisation of protein expression from microbial expression systems (Islam *et al.*, 2007; Holmes *et al.*, 2009), however such an approach has not been applied to lentiviral vector process development. Thus, the primary aim of this first results chapter is to develop a microwell experimental platform that can support suspension cultures of lentiviral vector producer cells, and to evaluate its utility (in conjunction with DoE techniques) for the rapid screening of multiple operating parameters to identify those most affecting titres. As it is important that any data generated using the microwell platform should remain relevant upon scale-up to larger vessels, engineering scaling parameters were considered. Mixing time (see Section 1.5.3) was chosen for evaluation as the utility of this criterion for scale-up of a mammalian cell culture process from a shaken microwell to a WAVE bioreactor (the vessel format that will ultimately be trialled for lentiviral vector

production in this work- see Chapter 5) has previously been demonstrated (Gill *et al.*, 2011). Throughout this chapter, the ProSavin® production process (based on suspension cultures of PS46.2) will be utilised as a model system (see Sections 1.3.3 and 1.4.5). The specific objectives of this chapter are:

- To evaluate a shaken 24-well plate system for the suspension culture of HEK293T-derived lentiviral vector producer cells (PS46.2), and to establish production methods compatible with this format.
- To assess whether mixing time could form an appropriate basis for scale-up of the lentiviral vector production process from a shaken microwell to a WAVE bioreactor.
- To demonstrate the utility of the microwell platform when combined with a DoE-guided approach, for the rapid screening of multiple operating parameters to ascertain their relative impact on lentiviral vector (ProSavin®) titre.

Results included in Section 3.2.5 of this chapter were published in: Guy, H. M., McCloskey, L., Lye, G. J., Mitrophanous, K. A. and Mukhopadhyay, T. K. (2013). Characterization of Lentiviral Vector Production Using Microwell Suspension Cultures of HEK293T-Derived Producer Cells. *Human Gene Therapy Methods* **24(2)**:125-139. Permission to reproduce this content has been granted by Mary Ann Liebert, Inc.

3.2 Results

3.2.1 Evaluation of a shaken microwell system for suspension culture of ProSavin® producer cells (PS46.2)

The first objective of this work was to evaluate whether shaken microwells could support suspension cultures of PS46.2. The microwell format chosen for evaluation was a standard 24 round well ultra-low attachment plate (Corning Inc.), for which utility (as assessed by the successful culture of murine hybridoma and CHO cell lines) had been previously demonstrated (Micheletti *et al.*, 2006; Barrett *et al.*, 2010; Silk *et al.*, 2010). Although smaller microwell culture formats exist, such as the 96-well shaken system (0.2 mL working volume) employed by Deshpande *et al.* (2004) for cultivation of CHO cells, here a working volume of ≥ 0.5 mL was required for analytical purposes. To minimise evaporation the plate was sealed using a

sandwich lid (Duetz *et al.*, 2000) in conjunction with a metal clamp as previously described (Silk *et al.*, 2010). Silk *et al.* (2010) reported that use of this cover limited the rate of fluid loss to 3 % day^{-1}, making it the preferred choice over the Diversified Biotech Breathe-Easy membrane (Sigma-Aldrich) that was employed during the earlier microwell studies conducted by Micheletti *et al.* (2006) and Barrett *et al.* (2010). The reported rate of fluid loss when using this cover was 6.3 % day^{-1} (Silk *et al.*, 2010).

Process development studies based on the PS46.2 cell line had, until this point, ordinarily been performed in shake flasks. It was desirable for future process development studies to be performed in microwells, rather than shake flasks, due to the associated cost savings and increased opportunity for parallelisation and thus experimental throughput. Standard shake flask cultures of PS46.2 (50 mL working volume) were seeded in parallel to microwell cultures (0.8 mL working volume), to provide a set of reference data against which the microwell results could be compared. Samples were removed from both vessels up to 189 hr post-seeding to enable analysis of the following parameters: viable cell concentration, cell viability, pH, osmolality, and concentration of metabolites (glucose, lactate, glutamine and ammonium). At each sampling point a small volume (~ 1 mL) of culture was removed from triplicate shake flasks, while triplicate microwell cultures were sacrificed. The initial choice of microwell operating conditions was based on established shake flask procedures for PS46.2 (Oxford BioMedica) and on previously published methods relating to the cultivation of mammalian cells using the elected microwell format (Micheletti *et al.*, 2006; Barrett *et al.*, 2010; Silk *et al.*, 2010). For a detailed description of the methods used see Section 2.2.1.

The primary objective of this study was to ascertain whether microwells could support comparable viable cell concentrations to shake flasks. For this purpose, comparability was defined according to whether a similar viable cell concentration (i.e. within ± 1 SD) was reached at the same time point in both vessels. All other parameters were monitored to gain greater insight into the growth dynamics of PS46.2 within each vessel, and were of secondary interest. For these parameters, comparability was determined according to whether the data for the two systems exhibited the same trend. Data was not subjected to statistical testing (e.g. ANOVA) as this was deemed inappropriate due to the small number of replicates involved ($n \leq 3$).

3.2.1.1 Cell growth and viability

As illustrated in Figure 3.1, the growth profiles of PS46.2 cultured in both shake flask and microwell formats were comparable, as similar peak viable cell concentrations (within ± 1 SD) were reached at the same time point. In both cases, the highest viable cell concentrations were observed on the third day of culture at 73 hr post-seeding, when densities reached 3.3 x 10^6 cells mL^{-1} in shake flasks and 4.0 x 10^6 cells mL^{-1} in microwells. There were differences in cell viabilities, however, which tended to be higher for shake flasks than microwells. This was most obvious at 73 hr post-seeding when the viability of shake flask cultures was 93.3 %, while the viability of microwell cultures was 73.3 %. As indicated by the fact that viable cell concentrations were similar, the *total* (viable and dead) cell concentration was markedly lower in shake flasks than microwells at this time point (3.5 x 10^6 cells mL^{-1} *versus* 5.4 x 10^6 cells mL^{-1}, respectively). This implies that microwell cultures of PS46.2 had a faster growth rate, and therefore it is possible that some factor became limiting to cell survival sooner for these cultures - leading to the observed divergence in cell viability between the two vessel types. Some dissimilarity between shake flasks and microwells in terms of cell growth was expected as the physical environment within each vessel is not identical (this will be discussed in Section 3.2.4). In addition, no attempt was made to improve comparability between the two systems as the initial cell growth data was deemed sufficiently similar as to satisfy the purpose of the study, i.e. it could be concluded that the microwell system was capable of supporting similar cell densities to shake flasks and further evaluation of the system, with a view to its application for early process development work, was therefore warranted.

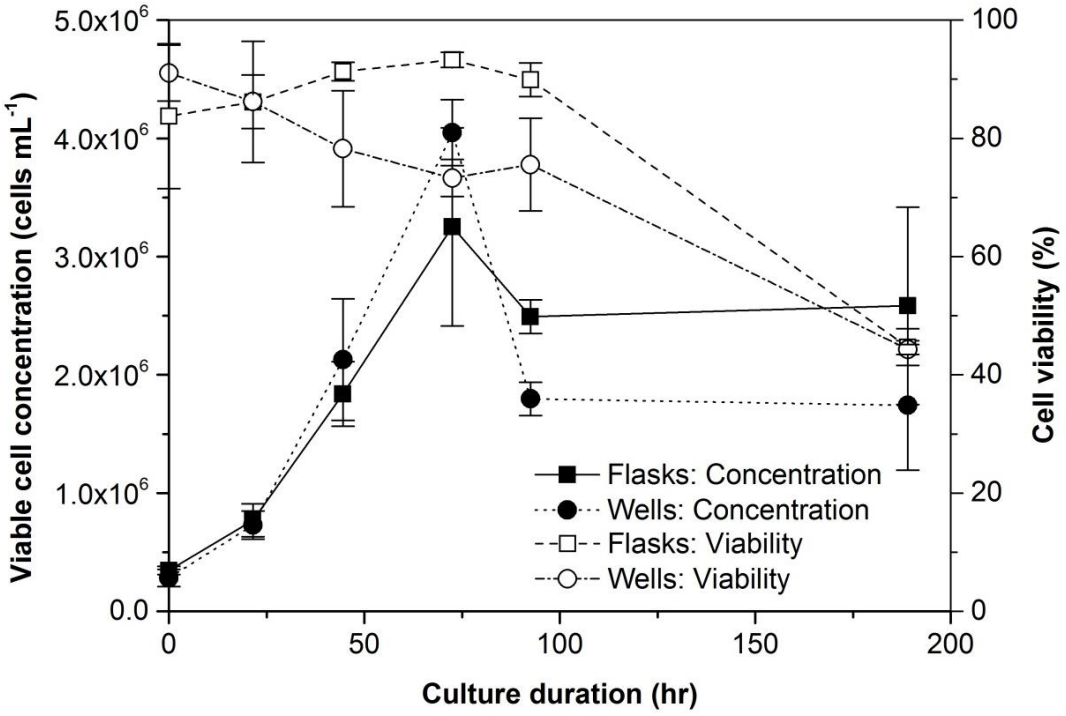


Figure 3.1 Comparison of parallel shake flask and microwell PS46.2 cultures in terms of cell growth and viability (n = 3). Shake flasks and microwells were seeded with PS46.2 at a density of 4 x 10^5 viable cells mL^{-1} in medium containing 5 % foetal calf serum (FCS). The orbital shaking diameter was 10 mm, while the shaking speed was maintained at 150 rpm for shake flasks and 180 rpm for microwells. Mean data is plotted and error bars represent ± one standard deviation (SD).

3.2.1.2 pH and osmolality

In order to better understand the observed growth dynamics of PS46.2; pH, osmolality, and metabolite data (collected at the same time as growth data) were also analysed. The normal pH of media designed to support mammalian cell cultures ranges from 6.8 - 7.5 (Brusick, 1986). During batch cultivations pH typically decreases over time as lactic acid is released as a by-product of cell metabolism (Naciri *et al.*, 2008). In the present study, the starting pH of the culture medium was 7.5 for both shake flask and microwell cultures (Figure 3.2). Consistent with an actively dividing and thus respiring cell population, the pH steadily decreased until 73 hr post-seeding, when values of 6.9 (shake flasks) and 7.0 (microwells) were recorded, corresponding with peak viable cell densities. As was expected, the decrease in pH was consistent with increasing lactate concentrations (Figure 3.3). As the pH did not fall appreciably

below 7.0 in either culture vessel, it is unlikely that culture acidification was responsible for the observed decline in viable cell numbers after 73 hr. From 73 hr until the end of the culture period the pH again rose (in-line with diminishing lactate concentrations) and at 189 hr values of 7.3 (shake flasks) and 7.5 (microwells) were recorded. Overall, the pH profiles for shake flask and microwell cultures were similar (Figure 3.2).

When handling low volumes, evaporation is a well-recognised concern (Berthier *et al.*, 2008), particularly with regards to cell culture (Girard *et al.*, 2001; Deshpande *et al.*, 2004) where it can cause elevated osmolalities and as a consequence potentially compromise cell health (Silk *et al.*, 2010). In the current study, the osmolality of shake flask cultures was 0.290 osmol kg^{-1} at the start of the cultivation period and declined marginally during the culture, measuring 0.267 osmol kg^{-1} after 189 hr (Figure 3.2). The osmolality of microwell cultures once seeded (0 hr) was similar to shake flasks (0.288 osmol kg^{-1}), but this value rose slightly throughout the cultivation period, reaching 0.316 osmol kg^{-1} by 189 hr. The higher osmolality in microwells as compared to shake flasks was expected based on the findings of Silk *et al.* (2010), who reported that although more liquid was lost from shake flask cultures than microwell cultures (310 µL day^{-1} *versus* 24 µL day^{-1}, respectively), this accounted for a much smaller proportion of the total starting volume (just 0.6 % *versus* 3 %, respectively). Thus assuming evaporation occurred to a similar extent in the current study, which is reasonable given that the vessels and fill volumes used were identical and the operating conditions were also alike, a greater rise in culture osmolality as a result of evaporation was to be anticipated for microwell cultures as compared to shake flask cultures. The observed increase in microwell osmolality is unlikely to have negatively impacted on PS46.2 cell growth. Mammalian cell cultures are normally maintained at an osmolality of around 300 osmol kg^{-1} (Hu and Aunins, 1997). Shen and Kamen (2012) reported that for suspension cultures of HEK293, raising the media osmolality from its standard level of 0.290 osmol kg^{-1} to 0.330 osmol kg^{-1} (higher than in the present study) had no detrimental effect on cell growth, however, raising the osmolality further to 0.370 osmol kg^{-1} reduced growth rates to around 80 % of those observed for standard cultures. Overall, the increase in microwell osmolality observed was small, and evaporation does not present a major concern when considering further development work when using this microwell platform in conjunction with the PS46.2 cell line - a typical ProSavin® production run lasts around 48 hr

(Section 2.2.2), over which period microwell osmolalities are unlikely to be significantly raised as compared to starting values (here, microwell osmolality was 0.298 osmol kg^{-1} after 45 hr cultivation). If the microwell platform were to be used for significantly extended cultivation periods, the addition of diluted bolus feeds could be considered as a means to offset fluid losses due to evaporation, as described by Silk *et al.* (2010).

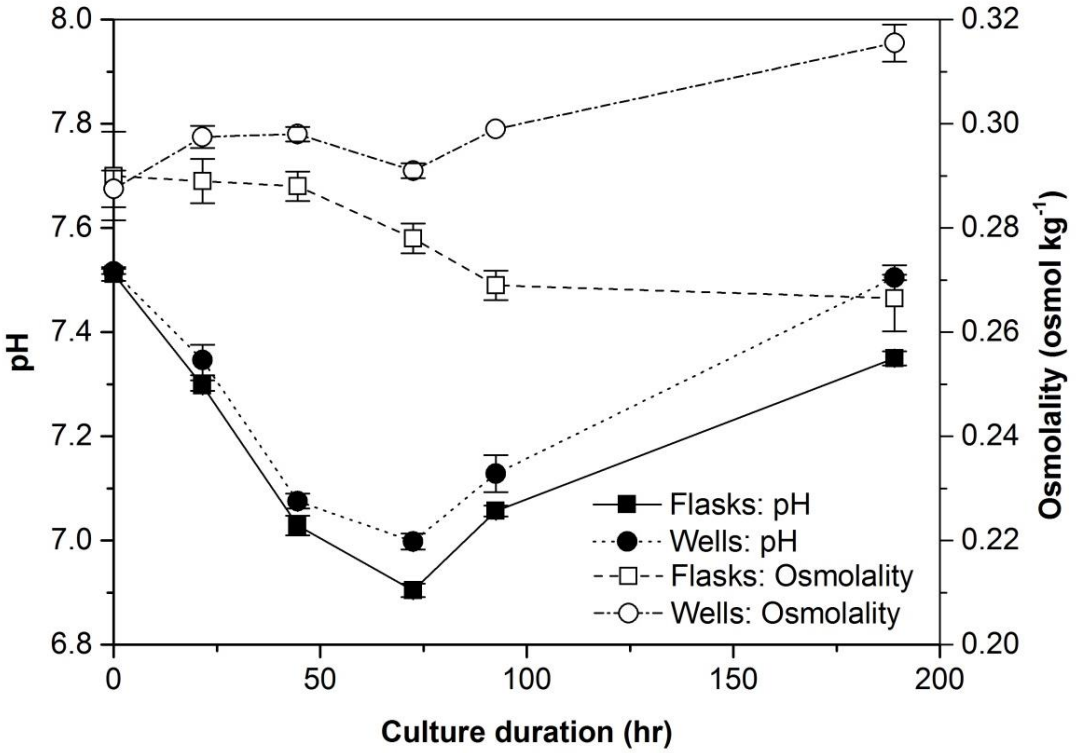


Figure 3.2 Comparison of parallel shake flask and microwell PS46.2 cultures in terms of pH and osmolality (n = 3). Operating conditions were as stated for Figure 3.1. Mean data is plotted and error bars represent ± 1 SD.

3.2.1.3 Glucose and lactate

The concentrations of glucose, lactate, glutamine, and ammonium in the culture medium were also monitored. Batch-cultured mammalian cells exhibit an inefficient metabolic phenotype that is characterised by the incomplete oxidation of glucose via the glycolytic pathway (the end product, pyruvate, is reduced to lactate, facilitating the oxidation of nicotinamide adenine dinucleotide [NADH] to NAD+ for re-use in glycolysis) and the incomplete oxidation of glutamine

via the glutaminolytic pathway (yielding ammonia and non-essential amino-acids) (Schneider *et al.*, 1996; Elias *et al.*, 2003; Quek *et al.*, 2010). A lack of glucose or glutamine, or an accumulation of ammonia or lactate, can contribute to cell death (Krampe and Al-Rubeai, 2010), thus to better understand the observed growth profiles for PS46.2 (Section 3.2.1.1) the concentrations of these key metabolites were monitored throughout the culture period.

As expected, the concentration of glucose in the culture medium decreased over time for both shake flask and microwell cultures (Figure 3.3). In shake flasks the glucose concentration was 2.7 g L^{-1} at the start of the culture (0 hr) but just 0.3 g L^{-1} at the end (after 189 hr). Values for microwell cultures were similar: the glucose concentration was 3.0 g L^{-1} at the time of seeding (0 hr) but after 189 hr only 0.2 g L^{-1} remained. The glucose supply was not exhausted in either vessel, however, and glucose is unlikely to have become a limiting nutrient at any point during the culture. Siegwart *et al.* (1999) previously reported no difference in the growth rate of bioreactor suspension cultures of HEK293 when a either a starting glucose concentration of 21 mM (3.8 g L^{-1}) was used or the glucose concentration was controlled at just 1 mM (0.2 g L^{-1}), and also noted that no growth lag occurred when shifting the glucose concentration from 21 to 1 mM for the initiation of the low-glucose controlled cultures. In the current work, following a peak in viable cell concentration 73 hr post-seeding, by 93 hr cell numbers had declined in both shake flasks and microwells (Figure 3.1), at which time a reasonable supply of glucose was still present in the culture medium (values of 0.7 g L^{-1} and 1.0 g L^{-1} were recorded for shake flask and microwell cultures, respectively). Glucose limitation is therefore unlikely to have instigated the drop off in viable cell numbers recorded at 93 hr, and thus the impact of other metabolites on the observed growth kinetics must be considered.

In both vessel types the observed reduction in available glucose over time was concurrent with an accumulation of lactate between 0 and 73 hr post-seeding (Figure 3.3). Over this period the lactate concentration in shake flasks increased from 0.2 g L^{-1} (0 hr) to 1.6 g L^{-1} (73 hr), and in microwells rose from 0.3 g L^{-1} (0 hr) to 2.2 g L^{-1} (73 hr). Lactate concentrations in excess of around 20 - 30 mM (1.8 - 2.7 g L^{-1}) have been reported to negatively impact on mammalian cell growth (Reuveny *et al.*, 1986; Ozturk *et al.*, 1992; Nadeau *et al.*, 1996; Schneider *et al.*, 1996; Cruz *et al.*, 2000; Patel *et al.*, 2000). Here, the concentration of lactate reached potentially inhibitory levels in microwells, but not in shake flasks. It has been well documented that for

non-pH controlled systems the reduction in extracellular pH associated with raised lactate levels is often the predominant factor responsible for slowed cell growth rates or decreased viabilities (Omasa *et al.*, 1992; Ozturk *et al.*, 1992; Schneider *et al.*, 1996; Patel *et al.*, 2000). In this study, although the culture pH declined in-line with the increasing lactate levels, significant culture acidification was not observed (Figure 3.2, Section 3.2.1.2). Overall, it therefore seems unlikely that accumulation of lactate instigated the decline in viable cell numbers observed in both vessels post-73 hr.

Finally, it is important to note that lactate concentrations declined after 73 hr, and by 189 hr values of 0.4 g L^{-1} (shake flasks) and 0.6 g L^{-1} (microwells) were recorded (Figure 3.3). For both vessel types, the fall in lactate concentration was concurrent with an increase in pH (Figure 3.2). The capacity of mammalian cells to switch to lactate consumption during the later phases of cell culture has been documented (Siegwart *et al.*, 1999; Altamirano *et al.*, 2006; Ma *et al.*, 2009; Li *et al.*, 2012; Mulukutla *et al.*, 2012). Lactate consumption is obviously desirable as its accumulation is reversed and its cytotoxic impact thus moderated, and during the production of recombinant proteins at least, a switch to lactate consumption during the later stages of culture has been strongly linked to an increase in cell productivity (Mulukutla *et al.*, 2012). It is unclear what triggered the metabolic shift to lactate consumption – it was unlikely induced by a lack of glucose, as this substrate remained available in reasonable quantities throughout the culture period.

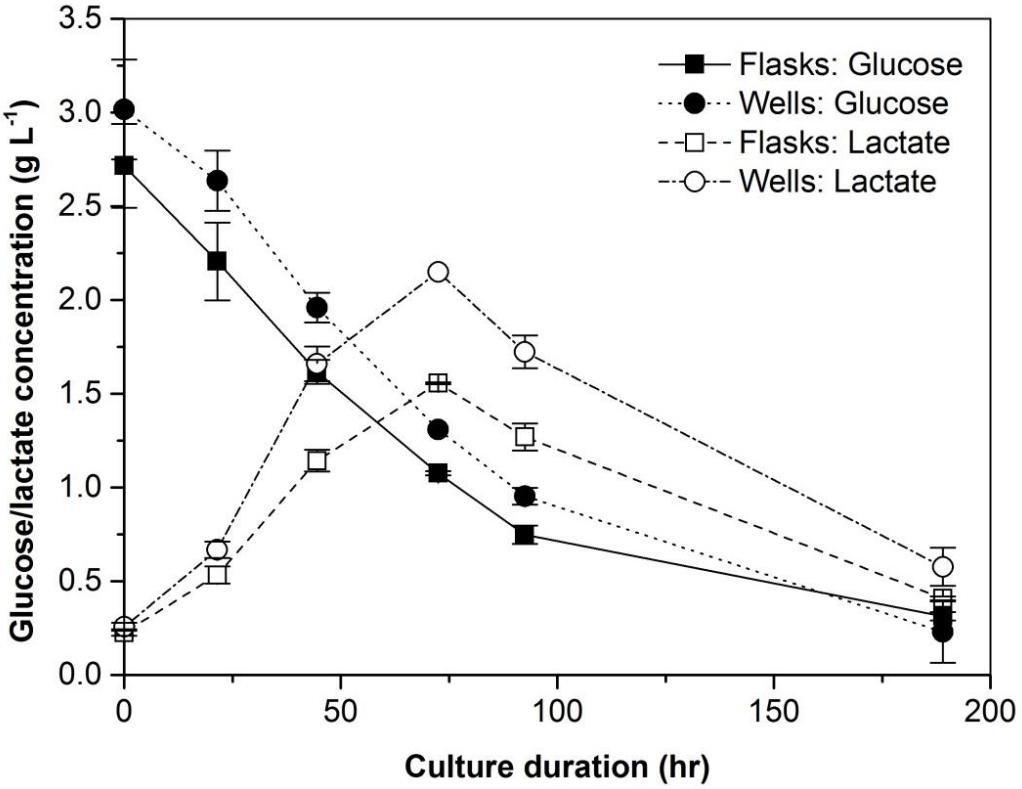


Figure 3.3 Comparison of parallel shake flask and microwell PS46.2 cultures in terms of glucose and lactate concentrations (n = 3 for all points except the 73 hr microwell sample, for which n = 1). Operating conditions were as stated for Figure 3.1. Mean data is plotted and error bars represent ± 1 SD.

3.2.1.4 Glutamine and ammonium

As well as containing glucose, the FreeStyle™ 293 expression medium used in this work contained GlutaMAX™ (Invitrogen) as a glutamine supplement. Although glutamine supplementation is essential for successful mammalian culture (Quek *et al.*, 2010), it spontaneously degrades to release ammonia which can build-up in the medium and have a cytotoxic impact (Hassell *et al.*, 1991). GlutaMAX™ is a dipeptide and stable precursor to glutamine, which is only hydrolysed to glutamine and alanine in the presence of peptidase (which is released from mammalian cells during culture), thus when using GlutaMAX™ the amount of ammonia produced via the chemical degradation of glutamine is reduced (Quek *et al.*, 2010). In this study, the glutamine concentration peaked 22 hr post-seeding when values of 0.27 g L^{-1} (shake flasks) and 0.31 g L^{-1} (microwells) were recorded, indicating that GlutaMAX™ was quickly hydrolysed once in contact with cells (Figure 3.4). After 93 hr glutamine levels in

shake flasks and microwells had declined to 0.10 g L^{-1} and 0.14 g L^{-1}, respectively. Siegwart *et al.* (1999) previously reported that when glutamine concentrations were below 1.6 mM (0.23 g L^{-1}) the growth rate of suspension-grown HEK293 slowed by 3 - 4 fold, and at concentrations less than 1.0 mM (0.15 g L^{-1}) the cells ceased to consume glutamine at all, although viabilities remained high for several days, and the authors suggested that ammonia may have been utilised as an alternative nitrogen source during this period. Based on the findings of Siegwart *et al.* (1999) by 73 hr glutamine levels were probably limiting in the current study, and by 93 hr glutamine may no longer have been being metabolised. Consistent with this hypothesis is the observation that viable cell numbers declined post-73 hr (Figure 3.1). In addition, from 93 hr to 189 hr glutamine levels again rose in shake flask and microwell cultures to 0.17 g L^{-1} and 0.22 g L^{-1}, respectively, implying that although hydrolysis of GlutaMAX™ continued during this period, the glutamine released was no longer consumed to the same extent as earlier in the culture.

Throughout the culture period, ammonium steadily accumulated in the medium of shake flasks and microwells (Figure 3.4). Build-up of ammonia is undesirable during mammalian cell culture as it perturbs electrochemical gradients and causes intracellular acidification, and energy consumption increases as the cells strive to maintain pH homeostasis; it can also induce apoptosis (Cruz *et al.*, 2000). Although the extent to which ammonia is tolerated depends on the specific cell line in question (Mirabet *et al.*, 1997), concentrations as low as 2 mM (0.04 g L^{-1}) have been reported to significantly impair mammalian cell growth (Reuveny *et al.*, 1986; Ozturk *et al.*, 1992; Schneider *et al.*, 1996; Cruz *et al.*, 2000; Henry and Durocher, 2011; Rajendra *et al.*, 2011). The toxic effects of ammonia are thus apparent at approximately ten-fold lower concentrations than those of lactate. In the current study, an ammonium concentration of 0.04 g L^{-1} was reached after just 45 hr culture for both vessel types, and final concentrations recorded after 189 hr were two to three-fold higher at 0.09 g L^{-1} (shake flasks) and 0.12 g L^{-1} (microwells). As ammonium concentrations did not fall at any point, it seems unlikely that ammonia was utilised as a nitrogen source when glutamine became scarce, as was noted previously during the batch culture of suspension-based HEK293 cells (Siegwart *et al.*, 1999). Considering the relatively high ammonium concentrations observed in this study, it is reasonable to suppose that this compound had some cytotoxic impact on PS46.2, particularly during the latter stages of the culture period, and may have contributed to the observed decline

in viable cell numbers post-73 hr. Finally, it is worth highlighting here the work of Cruz *et al.* (2000), who found that stirred cultures of BHK cells were more susceptible than stationary cultures to the effects of elevated ammonia levels: 3.5 mM (0.06 g L^{-1}) reduced growth rates by 50 % in stationary cultures, however just 1.1 mM (0.02 g L^{-1}) effected the same inhibition in stirred cultures, while overall cell death was induced at concentrations above 7.6 mM (0.14 g L^{-1}) and 2.6 mM (0.05 g L^{-1}), respectively. The authors hypothesised that agitation may promote a higher uptake of ammonia by the cell (Cruz *et al.*, 2000), thus worth consideration is the potential sensitivity of suspension-adapted PS46.2 to elevated ammonia levels when cultured using shaken systems.

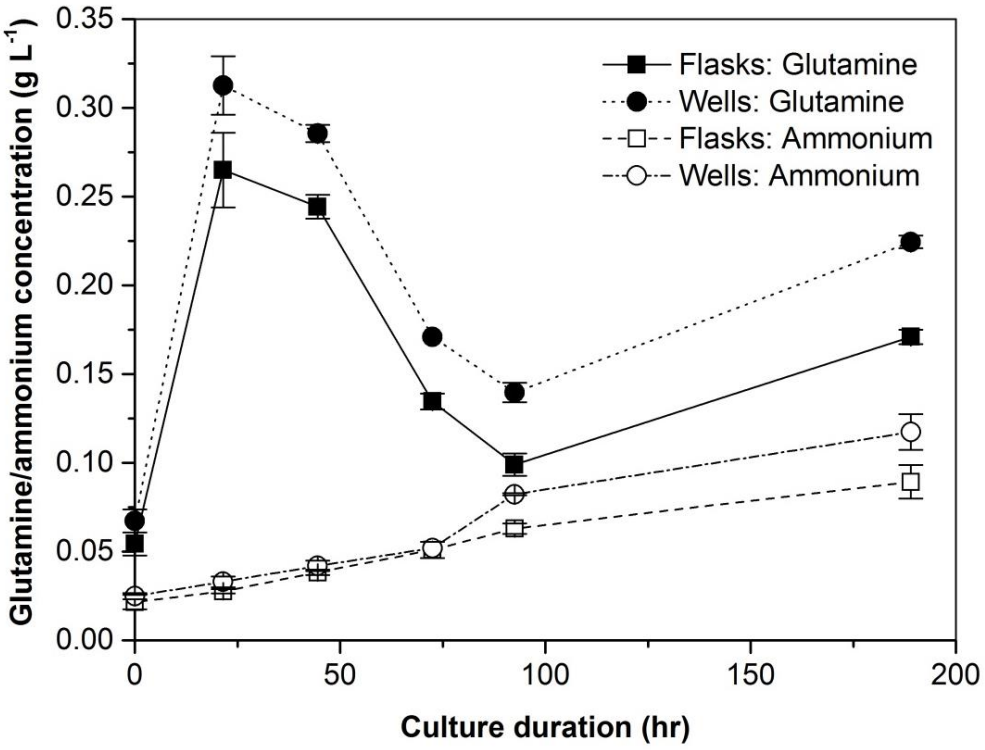


Figure 3.4 Comparison of parallel shake flask and microwell PS46.2 cultures in terms of glutamine and ammonium concentrations (n = 3 for all points except the 73 hr microwell sample, for which n = 1, and the 189 hr microwell sample [glutamine only], for which n = 2). Operating conditions were as stated for Figure 3.1. Mean data is plotted and error bars represent ± 1 SD.

3.2.2 ProSavin® production using the shaken microwell system

As the growth and associated culture kinetics of suspension-adapted PS46.2 cultured in shake flasks *versus* microwells were found to be comparable (Section 3.2.1), lentiviral vector production was next evaluated. Shake flasks and microwells were seeded in parallel and induced 24 hr later by the addition of 1.0 µg mL^{-1} dox and 10 mM NaBu. Harvests were performed 24 hr post-induction and titres determined using the rapid ProSavin® titre assay (Section 2.11.2.1). Note that the microwell and shake flask procedures were broadly similar, except that a 96-well filter plate was used to clarify microwell supernatants, as the syringe filter used for shake flask harvests was not appropriate for microlitre volumes (NB a separate study verified that the two filtration methods yielded comparable ProSavin® titre and RNA copy number results when a single batch of material was processed in parallel [data not shown]). Full details of the methods used are provided in Section 2.2.2. Shake flask titres were found to be 2.48 ± 0.73 x 10^4 TU mL^{-1} (mean ± 1 SD; n = 3), while microwell titres were 1.80 ± 0.43 x 10^4 TU mL^{-1} (mean ± 1 SD; n = 4). Microwell titres were within two-fold (and within ± 1 SD) of those recorded for shake flasks, thus, even without prior optimisation of the microwell process, reasonable comparability between the two vessel types was achieved. As cell growth kinetics and associated culture parameters were also found to be similar at the two culture scales (Section 3.2.1), taken together the data indicate that microwells could be used effectively in place of shakes flasks for early phase process development studies based on the PS46.2 cell line.

3.2.3 Application of the shaken microwell system for the suspension culture of a second (HEK293T) cell line

To verify the broader applicability of the microwell platform, the culture kinetics of a second HEK293T cell line (not stably integrated with lentiviral vector DNA) grown in shake flasks *versus* microwells were also examined. Shake flasks and microwells were seeded in parallel and samples were removed up to 189 hr post-seeding to enable analysis of viable cell concentration, cell viability, pH, osmolality, and concentration of metabolites (glucose, lactate, glutamine and ammonium). At each sampling point approximately 1 mL of culture was removed from triplicate shake flasks, while triplicate microwell cultures were sacrificed. The

methodology, which was analogous to that previously employed during the earlier evaluative study based on the PS46.2 cell line (for which the results were presented in Section 3.2.1), is described in full in Section 2.2.3. The only notable difference between the two studies (other than the choice of cell line), was that the culture medium used in this study contained 1 % foetal calf serum (FCS), whereas in the earlier study (Section 3.2.1) the culture medium contained 5 % FCS. The same criteria for comparability were used as described in Section 3.2.1.

3.2.3.1 Cell growth and viability

As illustrated in Figure 3.5, the growth profiles of HEK293T cultured in both shake flask and microwell formats were similar. Comparable maximum viable cell concentrations were reached in both vessels (within ± 1 SD). However, these were observed on the third day of culture (72 hr post-seeding) for shake flasks (2.6×10^6 cells mL^{-1}) and on the sixth day of culture (144 hr post-seeding) for microwells (2.2×10^6 cells mL^{-1}). An obvious peak in viable cell numbers was not observed in either vessel (Figure 3.5), however, and cell densities did not reach as high as those observed during the earlier culture of PS46.2 (Section 3.2.1.1, Figure 3.1). It is therefore likely that this particular HEK293T cell line was slower growing than PS46.2, and that peak cell densities were achieved on either the fourth or fifth day of culture when sampling was not conducted, thus this data was not captured in the current experiment. The viability of HEK293T cells in shake flasks and microwells remained comparable throughout the cultivation period (Figure 3.5).

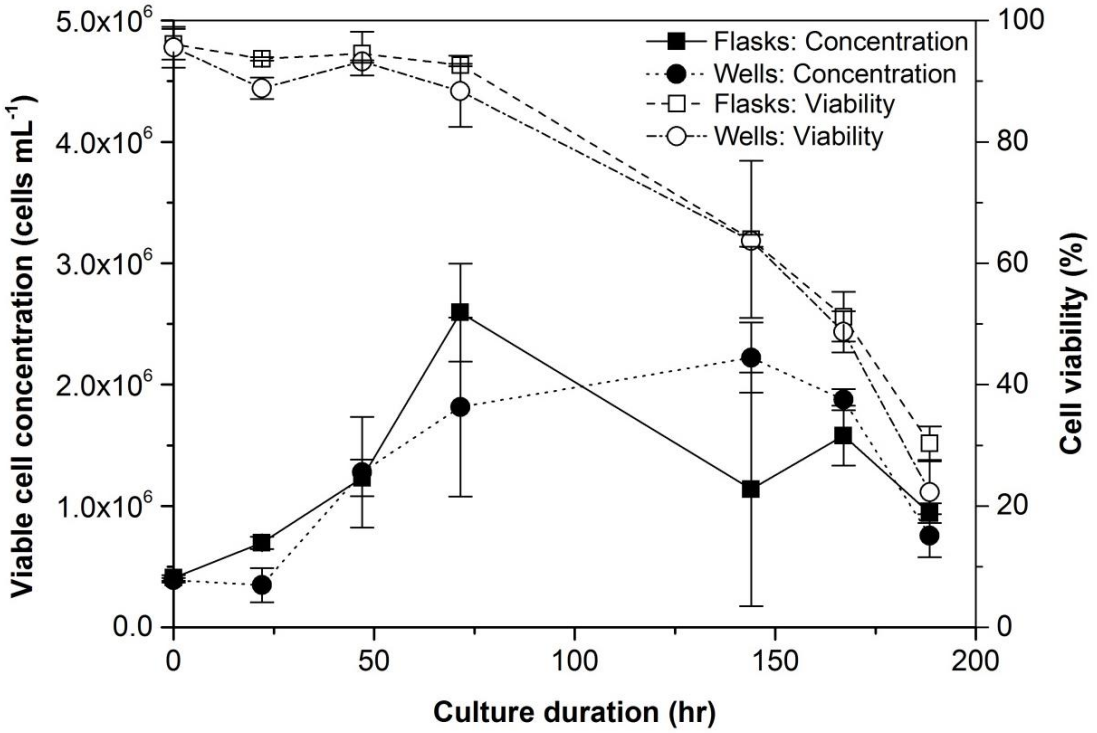


Figure 3.5 Comparison of shake flask and microwell HEK293T cultures in terms of cell growth and viability (n = 3). Shake flasks and microwells were seeded with HEK293T cells at a density of 4 x 10^5 viable cells mL^{-1} in medium containing 1 % FCS. The orbital shaking diameter was 10 mm, while the shaking speed was maintained at 150 rpm for shake flasks and 180 rpm for microwells. Mean data is plotted and error bars represent ± 1SD.

3.2.3.2 pH and osmolality

The starting pH of the culture medium was 7.6 for shake flask cultures and 7.5 for microwell cultures (Figure 3.6). The pH steadily decreased throughout the culture and after 189 hr the pH in shake flasks was ≤ 6.3 (a value of 6.3 was recorded for one replicate – the pH of the other two replicates was below the quantifiable limit [6.3] of the instrument) and in microwells was 6.4. The pH profiles for shake flask and microwell cultures were comparable. The decrease in pH was consistent with increasing lactate concentrations (Figure 3.7). At the 72 hr time point, the pH was 6.7 in both vessels, thus it is possible that culture acidification contributed to cell death. Note that the pH profile observed here (Figure 3.6) differed markedly to that observed earlier for the PS46.2 cell line (Section 3.2.1.2, Figure 3.2).

The osmolality of shake flasks varied little throughout the culture period, measuring 0.280 osmol kg^{-1} at the start (0 hr) and 0.297 osmol kg^{-1} at the end (189 hr post-seeding) (Figure 3.6). As expected, because of evaporation (see Section 3.2.1.2), the osmolality of microwell cultures, however, increased over time. Microwell osmolalities rose from 0.284 osmol kg^{-1} (0 hr) to 0.344 osmol kg^{-1} (189 hr). The 189 hr value is slightly higher than that previously recorded in microwells following 189 hr cultivation of PS46.2 (Figure 3.2), however, for lentiviral vector process development work, where it is anticipated that cultivations will be terminated much earlier than 189 hr, this does not present a concern (Section 3.2.1.2).

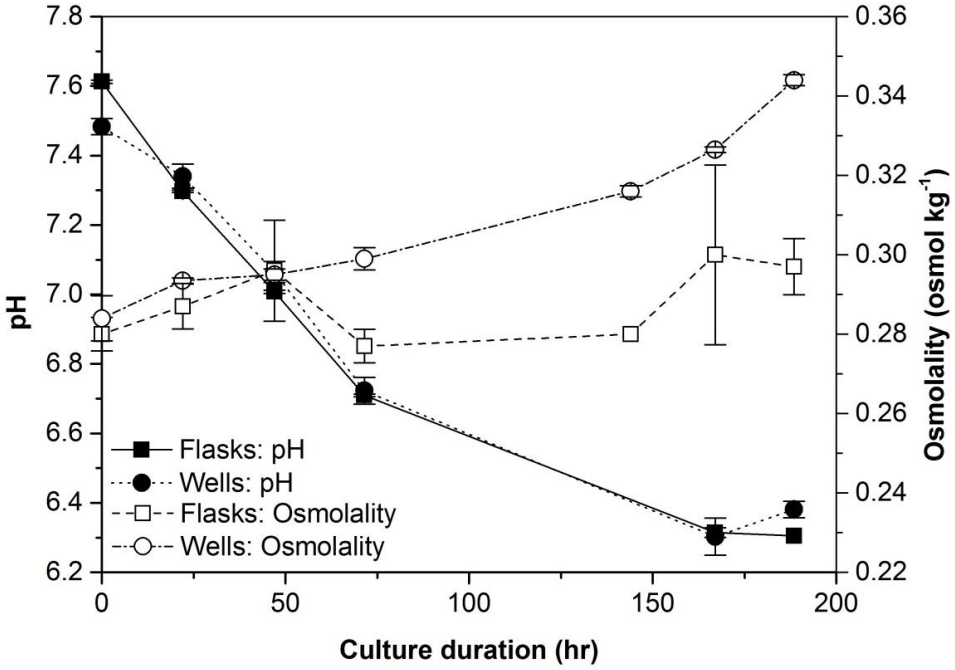


Figure 3.6 Comparison of parallel shake flask and microwell HEK293T cultures in terms of pH and osmolality (n = 3 for all points except the 189 hr shake flask pH sample, for which n = 1 as the pH values for the other two replicates were below the quantifiable limit [6.3] of the instrument). Operating conditions were as stated for Figure 3.5. Mean data is plotted and error bars represent ± 1 SD.

3.2.3.3 Glucose and lactate

The concentration of glucose in the culture medium declined over time for both shake flask and microwell cultures (Figure 3.7). In shake flasks the glucose concentration was 4.2 g L^{-1} at the

start of the culture (0 hr) but just 0.2 g L^{-1} at the end (after 189 hr). Values for microwell cultures were similar: the glucose concentration was 4.2 g L^{-1} at the time of seeding (0 hr) but after 189 hr only 0.1 g L^{-1} remained. It seems unlikely that glucose became a limiting nutrient for shake flask cultures (see discussion in Section 3.2.1.3), however, the possibility cannot be ruled out for microwell cultures.

In both vessel types the observed decline in glucose over time was concurrent with a build-up of lactate (Figure 3.7). The highest lactate values were observed on the sixth day of culture (144 hr post-seeding) for both shake flask (3.1 g L^{-1}) and microwell cultures (3.5 g L^{-1}). At the end of the culture (189 hr), lactate concentrations were still high, measuring 2.6 g L^{-1} in shake flasks and 3.2 g L^{-1} in microwells. It is likely that these elevated lactate levels produced the significant reduction in culture pH noted earlier (Figure 3.6), and thus were indirectly detrimental to cell health. Consequently, this cell line responded very differently to lactate accumulation than PS46.2, which was able to switch to lactate consumption after 73 hr culture and prevent culture acidification (Section 3.2.1.3, Figures 3.2 and 3.3). Parental and sub-clonal Chinese hamster ovary (CHO) cell lines have previously been demonstrated to display distinct lactate profiles, which the authors concluded was due to differences in their mitochondrial oxidative capacity (Zagari *et al.*, 2013). Thus, the metabolic response of any one cell line to changing environmental conditions will likely be unique, and conclusions relating to one cell line should not be generalised, even to other closely related clones.

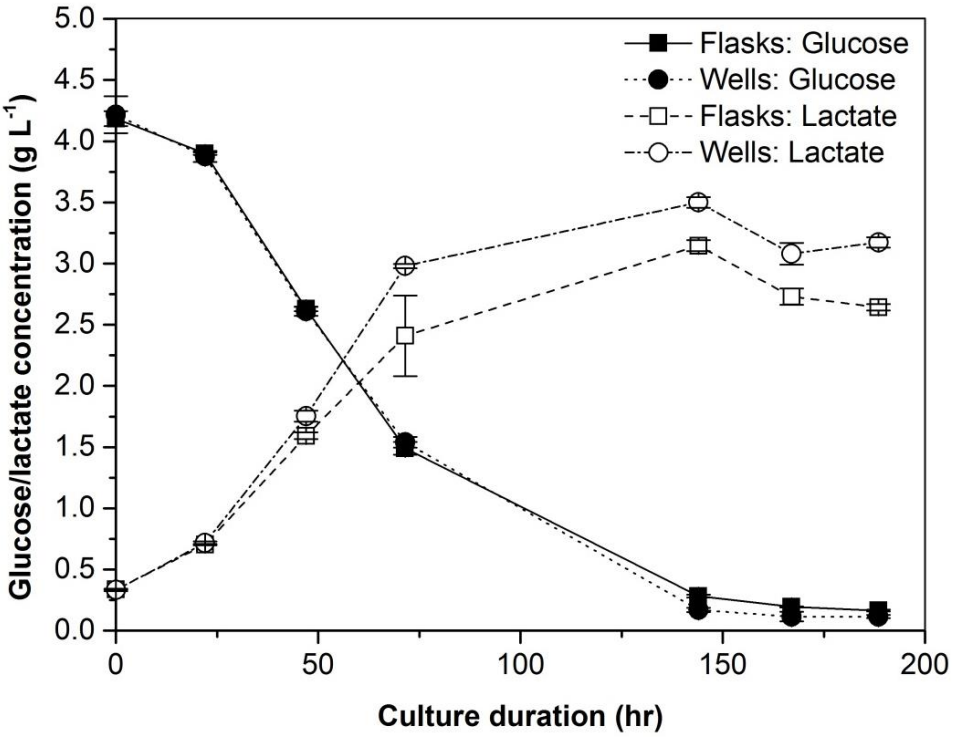


Figure 3.7 Comparison of parallel shake flask and microwell HEK293T cultures in terms of glucose and lactate concentrations (n = 3). Operating conditions were as stated for Figure 3.5. Mean data is plotted and error bars represent ± 1 SD.

3.2.3.4 Glutamine and ammonium

As noted earlier (Section 3.2.1.4), the FreeStyle™ 293 expression medium used in this work contained GlutaMAX™, which is hydrolysed to glutamine and alanine in the presence of peptidase that is released during cell culture. Here, as observed previously during the culture of PS46.2 cells (Figure 3.4), GlutaMAX™ was quickly hydrolysed once in contact with cells and glutamine concentrations peaked 22 hr post-seeding in both shake flasks and microwells, when values of 0.30 g L^{-1} and 0.32 g L^{-1} were recorded, respectively (Figure 3.8). By 72 hr, glutamine levels had fallen to 0.16 g L^{-1} (shake flasks) and 0.19 g L^{-1} (microwells), and were likely limiting in both vessels (see earlier discussion in Section 3.2.1.4). Unlike as was observed during the cultivation of PS46.2 (Figure 3.4), glutamine concentrations remained low until termination of the culture at 189 hr, at which point values of 0.05 g L^{-1} (shake flasks) and 0.04 g L^{-1} (microwells) were recorded (Figure 3.8), suggesting that the HEK293T cultures continued to metabolise glutamine throughout the culture period.

Throughout the culture period, ammonium gradually accumulated in shake flasks and microwells (Figure 3.8). Interestingly, at the start of the culture (0 hr), the concentration of ammonium was 0.05 g L^{-1} in shake flasks and 0.06 g L^{-1} in microwells, thus already at levels potentially inhibitory to cell growth (see discussion in Section 3.2.1.4). It is unclear why this should be the case when spontaneous degradation of glutamine to ammonia should be reduced in FreeStyle™ 293 expression medium due to the substitution of glutamine with GlutaMAX™ (a stable glutamine precursor). One explanation for this finding could be that peptidases present within the FCS used to supplement the culture medium hydrolysed the GlutaMAX™ and released glutamine, which subsequently degraded to ammonia, during storage of the supplemented medium (supplemented medium was stored at 4 °C for up to one month – however, as a precaution, for subsequent studies this was reduced to three weeks). Ammonium concentrations were two to three-fold higher by 189 hr, when concentrations of 0.11 g L^{-1} and 0.13 g L^{-1} were recorded in shake flasks and microwells, respectively (Figure 3.8). It is therefore probable that ammonia exerted a cytotoxic impact on HEK293T cells throughout the culture period. The ammonium profile observed here was similar to that previously observed for the PS46.2 cell line (Figure 3.4). Overall, the cell growth, pH, and metabolite profiles for shake flask and microwell HEK293T cultures were comparable, indicating that the cells responded to changing environmental conditions similarly in both vessels. This demonstrates that the utility of the microwell platform is not restricted solely to the PS46.2 cell line, but that its application may be extended to other HEK293T cell derivatives.

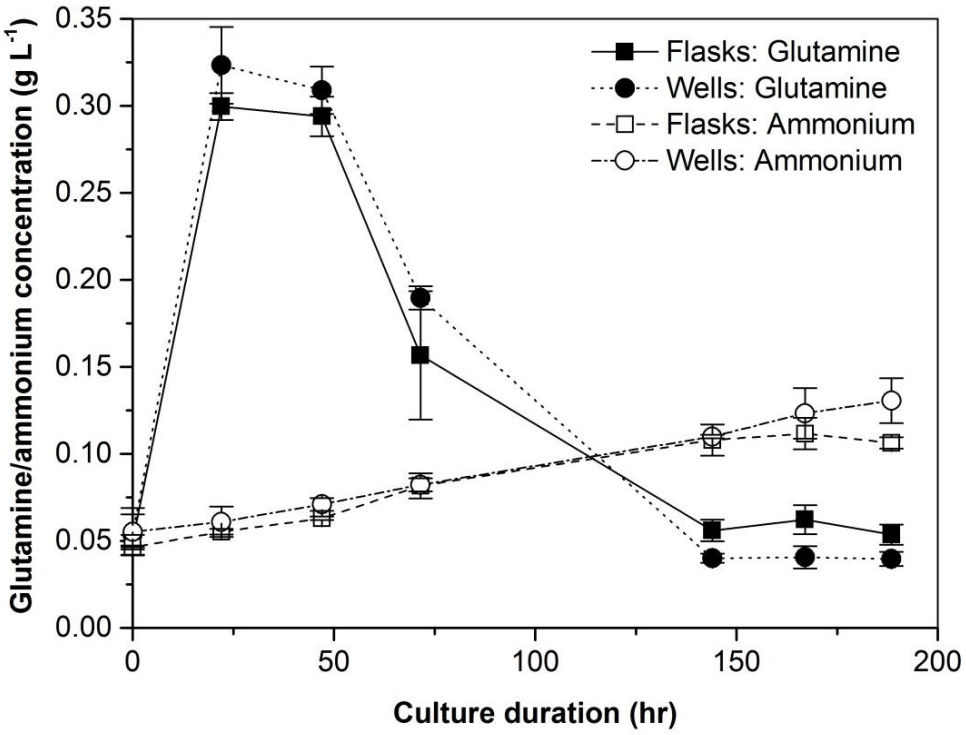


Figure 3.8 Comparison of parallel shake flask and microwell HEK293T cultures in terms of glutamine and ammonium concentrations (n = 3). Operating conditions were as stated for Figure 3.5. Mean data is plotted and error bars represent ± 1 SD.

3.2.4 Assessment of mixing time as a scaling criterion

The 24-well plate system trialled here successfully supported ProSavin® production based on suspension cultures of PS46.2 (Sections 3.2.1 and 3.2.2), making it an attractive low-volume alternative to shake flasks for early phase process development work. However, for the microwell platform to be usefully employed, any data generated should be relevant to larger scales (Micheletti *et al.*, 2006; Marques *et al.*, 2010). As discussed in Section 1.5.3, no universal strategy exists for bioprocess scale-up. The low oxygen requirements of mammalian cells (Micheletti *et al.*, 2006) mean that use of the oxygen mass transfer coefficient (k_La; refer to Section 1.5.3) as a scaling criterion is inappropriate while, on the other hand, the sensitivity of mammalian cells to shear forces (Kretzmer and Schügerl, 1991) mean that maintaining similar hydrodynamic conditions is likely crucial. Power per unit volume (*P/V*; refer to Section 1.5.3) directly relates to shear rate, and matched average *P/V* values have been used as a basis for scaling antibody production processes from microwells to shake flasks (Micheletti *et al.*, 2006;

Barrett *et al.*, 2010). However, this approach is unlikely to be effective for the translation of cell culture processes from shaken vessels to WAVE bioreactors. *P/V* within a microwell varies spatially (Barrett *et al.*, 2010), while in a WAVE bioreactor *P/V* varies both spatially and over time (as a direct function of the rocking motion) (Eibl *et al.*, 2009). Even if the overall average *P/V* for each system could be matched, the actual level of hydrodynamic stress experienced by cells is likely to be very different, thus scaling on the basis of a matched average *P/V* value is likely to be inappropriate in this instance. An alternative scaling criterion to *P/V* is mixing time. Mixing time has previously been used as a scaling criterion for the translation of an antibody production process from a shaken microwell with 850 µL working volume to a WAVE bioreactor with 1 L or 4 L working volume (Gill *et al.*, 2011). Encouraged by this report, the potential of mixing time as a scaling criterion for translation of the ProSavin® production process (as described in Section 3.2.2) from a shaken microwell to a WAVE bioreactor was assessed. Mixing time experiments were carried out for a shaken microwell and, for comparison, other small-scale culture vessels, using the iodine decolourisation method as described in Section 2.3.

Listed in Table 3.1 are the mixing time values obtained for a shaken microwell and various other small scale culture vessels. For a microwell operated under conditions initially found to yield acceptable cell growth kinetics and lentiviral vector titres (Sections 3.2.1 and 3.2.2; fill volume 800 µL and shaking speed 180 rpm), the mixing time was extremely long (1037 s). This is in agreement with the findings of Barrett *et al.* (2010), who reported that for a microwell with an 800 µL fill volume and *Re* of around 1000 (here, *Re* was calculated to be 1049), the mixing time was approximately 1000 s. As illustrated in Figure 3.9, there is little deformation of the fluid surface at this shaking speed and when a small volume of liquid is added (as during the mixing time experiments) this descends to the base of the microwell, where it spreads out and gradually rises and disperses within the bulk fluid (the time taken for complete homogeneity to be achieved is equivalent to the mixing time). This visualisation also mimics what would most probably happen during the addition of inducer compounds (dox and NaBu) during cell culture. It is evident that heterogeneities would exist temporarily, which may be of concern if these compounds exert a toxic influence at high concentrations (Barrett *et al.*, 2010). This concern is

exacerbated by the fact that microwells are typically stationary when such additions are made (microwells are transferred to a biological safety cabinet to preserve sterility).

Table 3.1 Mixing time values for various small scale culture vessels. The diameter of the shaking platform was 10 mm. Mixing times were determined using the iodine decolourisation method as outlined Section 2.3. Mean data is presented and error bars represent ± 1 SD (n = 3).

Vessel type	Total capacity (mL)	Working volume (mL)	Shaking speed (rpm)	Mixing time (s)
Microwell	3.4	0.8	140	1374 ± 30
Microwell	3.4	0.8	180	1037 ± 40
Microwell	3.4	0.8	220	333 ± 20
Microwell	3.4	0.8	260	15 ± 1
Shake flask	125	25	150	29 ± 4
Shake flask	250	50	150	24 ± 7
Shake flask	500	100	150	29 ± 3
Roller bottle	2000	4000	150	8 ± 1

As expected, the mixing time was slower when the shaking speed was decreased to 140 rpm, and faster when the shaking speed was increased to 220 and 260 rpm (Table 3.1). Eibl *et al.* (2010b) reported that mixing times for a WAVE bioreactor, when operated under various rocking conditions and with a standard 40 - 50 % fill volume, were in the range of 9 to 264 s for a 2 L system (the scale later evaluated in this work – see Chapter 5), and 65 to 874 s for a 200 L system (the scale hypothetically required for commercial production of ProSavin®). Thus, if the mixing time in microwells is to be reproduced in both a 2 L and 200 L WAVE bioreactor, a value of between 65 and 264 s is necessary, for which a platform shaking speed of between 220 and 260 rpm would be required (see Table 3.1). As preliminary experiments indicated that such conditions were unfavourable for PS46.2 cell growth (data not shown), the use of mixing time as a scaling criterion was deemed impracticable. Furthermore, the observation that the mixing time was around 40-fold shorter for a 250 mL shake flask shaken at 150 rpm (24 ± 7 s) as compared to a microwell shaken at 180 rpm, yet cell growth kinetics and lentiviral vector titres obtained using the two vessels under these operating conditions were equivalent (Sections 3.2.1 and 3.2.2), suggests that mixing time is not a good indicator of culture performance and thus its use as a scaling parameter is not appropriate here. Consequently, a DoE-guided approach was undertaken to identify the operating parameters which *do* impact on PS46.2

culture performance (Section 3.2.5), and their optimal ranges (Chapter 4). This information was used in conjunction with knowledge of the hydrodynamic environment favoured by cells (obtained by the calculation of dimensionless numbers) to inform the design of a large scale culture process, which was subsequently trialled (Chapter 5). Thus, rather than relying on a single parameter as the basis for scale-up studies, a more holistic approach was implemented.

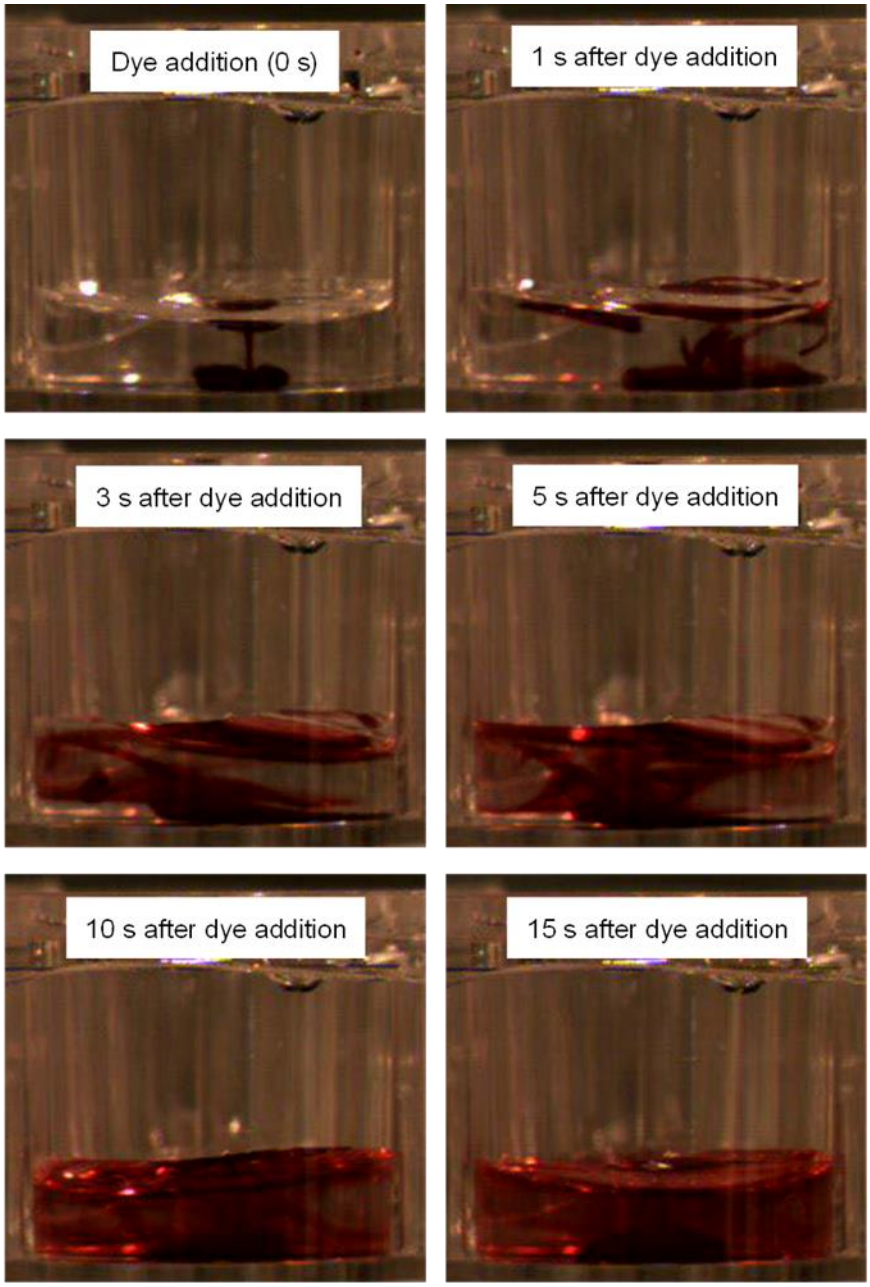


Figure 3.9 High speed video images of a small liquid addition (8 µL inert dye) made to a microwell containing 792 µL water. The microwell was mounted on a shaking platform of 10 mm diameter rotating at a speed of 180 rpm.

3.2.5 Microwell screening experiment to determine the relative impact of nine process parameters on ProSavin® titre

3.2.5.1 Results of the screening experiment

Having demonstrated that ProSavin® production (based on suspension cultures of PS46.2) could be successfully carried out using a 24-well plate system (Sections 3.2.1 and 3.2.2), a low-cost, high-throughput approach to bioprocess optimisation could now be implemented. However, as an appropriate criterion for scale-up was not yet identified (Section 3.2.4), the future translation of any improvements obtained at the microwell scale to larger vessels (preferably, WAVE bioreactors), remained a concern. Consequently, a microwell-based structured approach to bioprocess development was undertaken that had two aims: (i) to identify and optimise critical factors affecting ProSavin® production from the PS46.2 suspension cell line (as previously noted, current titres fell short of those required for clinical application – Section 1.4.5), and (ii) to use the acquired knowledge to inform the design of a large scale process. As a large number of parameters could potentially impact on ProSavin® production, it was logical to employ DoE methodology to explore their various effects. As discussed in Section 1.5.2, typically the first objective when implementing DoE is familiarisation, to determine what is experimentally possible (Eriksson *et al.*, 2008). This had been largely accomplished through the earlier work described in Sections 3.2.1 and 3.2.2 and through a handful of pilot studies (data not shown). This information, in conjunction with that derived from both published (Stevenson *et al.*, 2009; Stewart *et al.*, 2009; Stewart *et al.*, 2011) and unpublished (Oxford BioMedica) sources, was therefore used to aid the design of a screening experiment (the next step in the DoE process). In this experiment, the relative impact of nine factors (Table 3.2) on ProSavin® titres was assessed. A fractional factorial design was generated, which reduced the number of experimental runs to one sixteenth of those otherwise required for a full factorial design. Even with this reduction, 36 experimental runs still needed to be carried out and, as it was desirable for these to be performed in triplicate, the microwell platform proved indispensable. For a detailed overview of the methods employed see Section 2.4.1.

Table 3.2 Specification of the factors and settings investigated during the screening experiment. Dox is an abbreviation of doxycycline.

Factor (units)	Abbreviation	Settings		
		-1	0	1
Shaking speed (rpm)	x_1	120	160	200
Shaking diameter (mm)	x_2	10	20	30
Pre-induction period (hr)	x_3	2	24	46
Post-induction period (hr)	x_4	6	26	46
Liquid fill volume (µL)	x_5	800	1400	2000
Cell seeding density (viable cells mL^{-1})	x_6	4.0×10^5	1.2×10^6	2.0×10^6
Concentration of serum (% [v/v])	x_7	1	3	5
Concentration of dox (µg mL^{-1})	x_8	0.2	1.0	1.8
Concentration of NaBu (mM)	x_9	2	10	18

The design matrix for the screening experiment and results (expressed as normalised titre values) are presented in Table 3.3. A regression model was fitted to normalised titre data, which had undergone a natural log transformation, as described in Section 2.4.1.1. Only main terms that were significant at the level $p < 0.1$ were included in the model. The model yielded an $adjR^2$ of 0.63 and $predR^2$ of 0.58, indicating that 63 % of response variation was accounted for by the model, and 58 % of variation in response data was predicted by the model. As the difference between the $adjR^2$ and $predR^2$ was < 0.3 and the $predR^2$ value was ≥ 0.5 (but < 0.9), the model fit was deemed sufficiently accurate for the purposes of identifying main factor effects. Analysis of variance (ANOVA) statistics are presented in Table 3.4. The results show that the model was significant ($p < 0.0001$), and that post-induction period ($p < 0.0001$), liquid fill volume ($p = 0.0003$), and concentration of dox ($p = 0.0076$) all had a significant influence on ProSavin® titres. Post-induction period had the largest effect, accounting for 40.9 % of the observed response variation, followed by liquid fill volume (17.3 %) and concentration of dox (8.5 %). As illustrated by the coefficient plot (Figure 3.10), higher vector titres were achieved at the high settings of post-induction period (46 hr) and concentration of dox (1.8 µg mL^{-1}), and at the low setting of liquid fill volume (800 µL). Logically, this model is correct, as only runs with this combination of high and low settings for post-induction period, liquid fill volume and concentration of dox (runs 10, 11, 14 and 15) produced normalised titre values of ≥ 20 % (yielding values of 20, 84, 156, and 73 %, respectively; Table 3.3). The ANOVA results also revealed that the model 'lack of fit' was significant ($p = 0.0452$), and this is because around one third (33.4 %) of the observed variation in ProSavin® titre could not be accounted for by differences in those terms included in the model (post-induction period, liquid fill volume and

concentration of dox). Further exploration of the data using DoE software revealed that this unexplained response variation was mostly caused by the terms that had been excluded from the model, i.e. by the collective impact of the other six factors, plus various interaction effects. Interaction terms were not included in the model as factorial designs do not support their valid interpretation (interactions terms are confounded with each other and/or the main effects). When considered individually, the main and interaction terms that had been excluded from the model each made a minor contribution to the observed response variation as compared to those terms that were included in the model. Their combined influence, however, accounted for most (29.1 %) of the unexplained response variation, while curvature in the model (due to centre point data falling above or below that predicted by the factorial design points) accounted for 3.9 %. The remaining unexplained response variation (0.4 %) was caused by uncontrolled process variability (i.e. pure error). Therefore, lack of model fit seems, to a certain extent, inevitable when investigating a large number of factors using a reduced experimental design. Although clearly something to be aware of, a significant 'lack of fit' value should not, on its own, prevent use of the model, as the aim of screening is simply to identify the most critical factors influencing a system, and not to use the model in any sort of predictive capacity. Here, as the effects of post-induction period, liquid fill volume, and concentration of dox were clearly distinguishable over and above those of the other factors, the screening experiment successfully achieved its aim.

Table 3.3 Resolution IV fractional factorial design matrix for the screening experiment. Nine factors were evaluated over two levels (high/low) to ascertain their relative impact on ProSavin® titre, and four centre point experiments were included for estimation of pure error. Factors and their ranges were coded according to Table 3.2. Each run was performed in triplicate and mean titres values (expressed as a percentage of control shake flask titres – see Section 2.4.1) are presented.

Run	x_1	x_2	x_3	x_4	x_5	x_6	x_7	x_8	x_9	Titre (% of control)
1	-1	-1	-1	-1	-1	1	1	1	1	3.77
2	1	-1	-1	-1	-1	-1	-1	-1	-1	1.17
3	-1	1	-1	-1	-1	-1	-1	-1	1	3.16
4	1	1	-1	-1	-1	1	1	1	-1	0.44
5	-1	-1	1	-1	-1	-1	-1	1	-1	0.33
6	1	-1	1	-1	-1	1	1	-1	1	0.59
7	-1	1	1	-1	-1	1	1	-1	-1	1.64
8	1	1	1	-1	-1	-1	-1	1	1	5.34
9	-1	-1	-1	1	-1	-1	1	-1	-1	14.62
10	1	-1	-1	1	-1	1	-1	1	1	19.86
11	-1	1	-1	1	-1	1	-1	1	-1	84.19
12	1	1	-1	1	-1	-1	1	-1	1	7.42
13	-1	-1	1	1	-1	1	-1	-1	1	2.63
14	1	-1	1	1	-1	-1	1	1	-1	155.72
15	-1	1	1	1	-1	-1	1	1	1	72.70
16	1	1	1	1	-1	1	-1	-1	-1	6.96
17	-1	-1	-1	-1	1	1	-1	-1	-1	0.00
18	1	-1	-1	-1	1	-1	1	1	1	1.56
19	-1	1	-1	-1	1	-1	1	1	-1	1.19
20	1	1	-1	-1	1	1	-1	-1	1	0.55
21	-1	-1	1	-1	1	-1	1	-1	1	0.33
22	1	-1	1	-1	1	1	-1	1	-1	0.00
23	-1	1	1	-1	1	1	-1	1	1	2.63
24	1	1	1	-1	1	-1	1	-1	-1	0.00
25	-1	-1	-1	1	1	-1	-1	1	1	8.30
26	1	-1	-1	1	1	1	1	-1	-1	5.32
27	-1	1	-1	1	1	1	1	-1	1	5.14
28	1	1	-1	1	1	-1	-1	1	-1	4.69
29	-1	-1	1	1	1	1	1	1	-1	0.99
30	1	-1	1	1	1	-1	-1	-1	1	1.17
31	-1	1	1	1	1	-1	-1	-1	-1	0.99
32	1	1	1	1	1	1	1	1	1	8.50
33	0	0	0	0	0	0	0	0	0	4.87
34	0	0	0	0	0	0	0	0	0	7.06
35	0	0	0	0	0	0	0	0	0	10.44
36	0	0	0	0	0	0	0	0	0	9.84

Table 3.4 Analysis of variance (ANOVA) results of the regression model for normalised titre (transformed as described in Section 2.4.1.1). df is an abbreviation for 'degrees of freedom'. The percentage contribution of a given factor to the observed variation in the response was calculated as follows: [x_i sum of squares / corrected total sum of squares] x 100.

Source	Sum of squares	df	Mean square	F value	*p* value
Model	64.05	3	21.35	21.28	< 0.0001
Post-induction period (x_4)	*39.27*	*1*	*39.27*	*39.14*	*< 0.0001*
Liquid fill volume (x_5)	*16.64*	*1*	*16.64*	*16.58*	*0.0003*
Concentration of dox (x_8)	*8.14*	*1*	*8.14*	*8.11*	*0.0076*
Residual	32.11	32	1.00		
Lack of fit	*31.75*	*29*	*1.09*	*9.27*	*0.0452*
Pure error	*0.35*	*3*	*0.12*		
Corrected total	96.16	35			

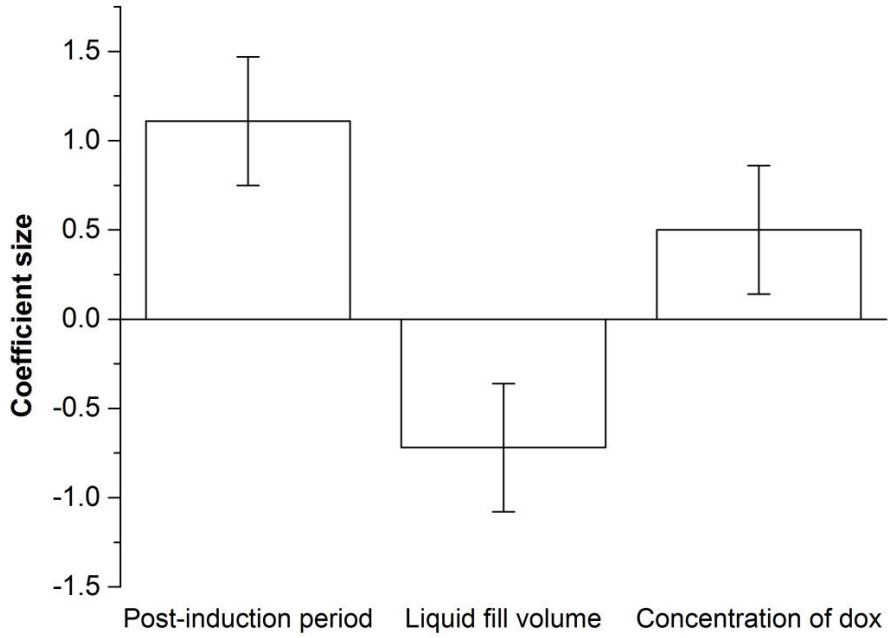


Figure 3.10 Coefficient plot of the regression model for normalised titre (transformed as described in Section 2.4.1.1), with confidence intervals set at 95 %. The size of each coefficient indicates the relative influence of that factor on titre. A positive coefficient indicates that an increase in that factor (relative to its centre point value) would result in an increase in titre, while for negative coefficients the reverse is true.

Here, a method combining DoE and microwell cultures was employed to rapidly identify the critical factors influencing the production of ProSavin® (an EIAV-derived lentiviral vector) from a suspension-adapted producer cell line (PS46.2). Out of nine factors, three were identified as having a significant influence on ProSavin® titre (Table 3.4) and together accounted for 66.6 %

of the observed response variation. Of these factors, post-induction period had the greatest impact, with higher titres being attained at the high setting of 46 hr (Figure 3.10). ProSavin® production is tightly regulated by the tet-ON system, and the addition of dox stimulates expression of both EIAV *gag/pol* and the cytotoxic VSV-G component (Stewart *et al.*, 2009; Stewart *et al.*, 2011). In PS46.2, particle assembly and budding can only proceed once Gag/Pol and Env proteins have accumulated to sufficient levels within the cell cytoplasm. As normalised titre values were extremely low for runs with a post-induction period of 6 hr (< 6 %; Table 3.3), supernatants were likely harvested before particle assembly was fully underway. For PS46.2 grown adherently, it was previously reported that a post-induction period of 22 hr yielded optimum titres (Stewart *et al.*, 2011). The influence of post-induction period on ProSavin® production using suspension cultures of PS46.2 will be examined in greater depth in Chapter 4.

Liquid fill volume had the second largest effect on titre, with higher titres being attained at the low setting of 800 µL (Figure 3.10). For the same 24-well plate format as employed here, it has been previously reported that when a shaking speed of 120 rpm and shaking diameter of 20 mm was used, a 2000 µL liquid fill volume led to poor oxygenation of mammalian cell cultures (k_La = 1.3 hr^{-1}; dissolved oxygen tension < 10 % between 60 and 96 hr culture) and lower cell densities, while an 800 µL fill volume did not (k_La = 3.2 hr^{-1}; dissolved oxygen tension > 60 % throughout the culture) (Barrett *et al.*, 2010). In the current study, runs conducted using a 2000 µL liquid fill volume produced normalised titre values of < 9 %, over a range of shaking conditions (Table 3.3). It was observed that cells tended to form aggregates that localised on the bottom of microwells (irrespective of the shaking conditions employed – see Figure 3.11, which is discussed in greater depth in Section 3.2.5.2) and, taken together with the findings of Barrett *et al.* (2010), it may be conjectured that an oxygen deprived microenvironment was created at the base of microwells operating with a 2000 µL fill volume (note that oxygen transfer is achieved through surface aeration only), which was unfavourable for ProSavin® production. Although cells also accumulated on the bottom of microwells operating with a 800 µL liquid fill volume (Figure 3.11), the rate of oxygen transfer within these cultures was likely superior (Barrett *et al.*, 2010), meaning that oxygen limited conditions were less likely to develop as a result.

Concentration of dox had the third largest effect on titre, with higher titres being attained at the high setting of 1.8 µg mL^{-1} (Figure 3.10). Normalised titre values were low (< 15 %) for all runs conducted at the low setting of 0.2 µg mL^{-1} (Table 3.3). For mammalian cells encoding a tet-ON regulatory system, dox has been shown to induce gene expression in a dose-dependent manner; the level at which maximum expression is attained varies according to the particular application, however values of 0.5 to 2.0 µg mL^{-1} appear typical (Koponen *et al.*, 2003; Tang *et al.*, 2009). PS46.2 encodes a tet-ON regulatory system, thus it is probable that a dox concentration of 0.2 µg mL^{-1} was insufficient for the full induction of cells in the current study. Induction of adherent cultures of PS46.2 is typically achieved using a dox concentration of 1.0 µg mL^{-1} (Stewart *et al.*, 2009; Stewart *et al.*, 2011). Obvious differences exist in the fluid hydrodynamics between shaken and static cell cultures thus, although a dox concentration of 1.0 µg mL^{-1} was initially adopted for microwell feasibility studies (Section 3.2.2), it was unknown whether this value was optimal - hence the inclusion of dox concentration as parameter in the screening experiment. From this experiment it may be concluded that lowering the dox concentration to 0.2 µg mL^{-1} is detrimental for ProSavin® titres, however, to identify its optimal operating range, this parameter, along with post-induction period and liquid fill volume, was consequently taken forward for further investigations as described in Chapter 4.

3.2.5.2 Impact of microwell operating conditions on cellular aggregation

The propensity for HEK293-derived cell lines to aggregate when cultured in suspension has been well documented (Wurm and Bernard, 1999; Liu *et al.*, 2006; Zhao *et al.*, 2007). During the screening experiment, it was observed that the size and arrangement of aggregates varied widely, depending on the specific combination of operating conditions employed. Although not photographed at the time, in an attempt to qualitatively capture this data, triplicate wells were later seeded according to each possible high/low combination of the factors considered most relevant (shaking speed, shaking diameter, liquid fill volume, cell seeding density and serum concentration of medium) using the parameter settings provided in Table 3.2. Cells were not induced, but were photographed 46 hr after seeding (as described in Section 2.4.1.4). Figure 3.11 shows a representative photograph from each set of conditions which, when viewed collectively, illustrate the general trends as discussed below.

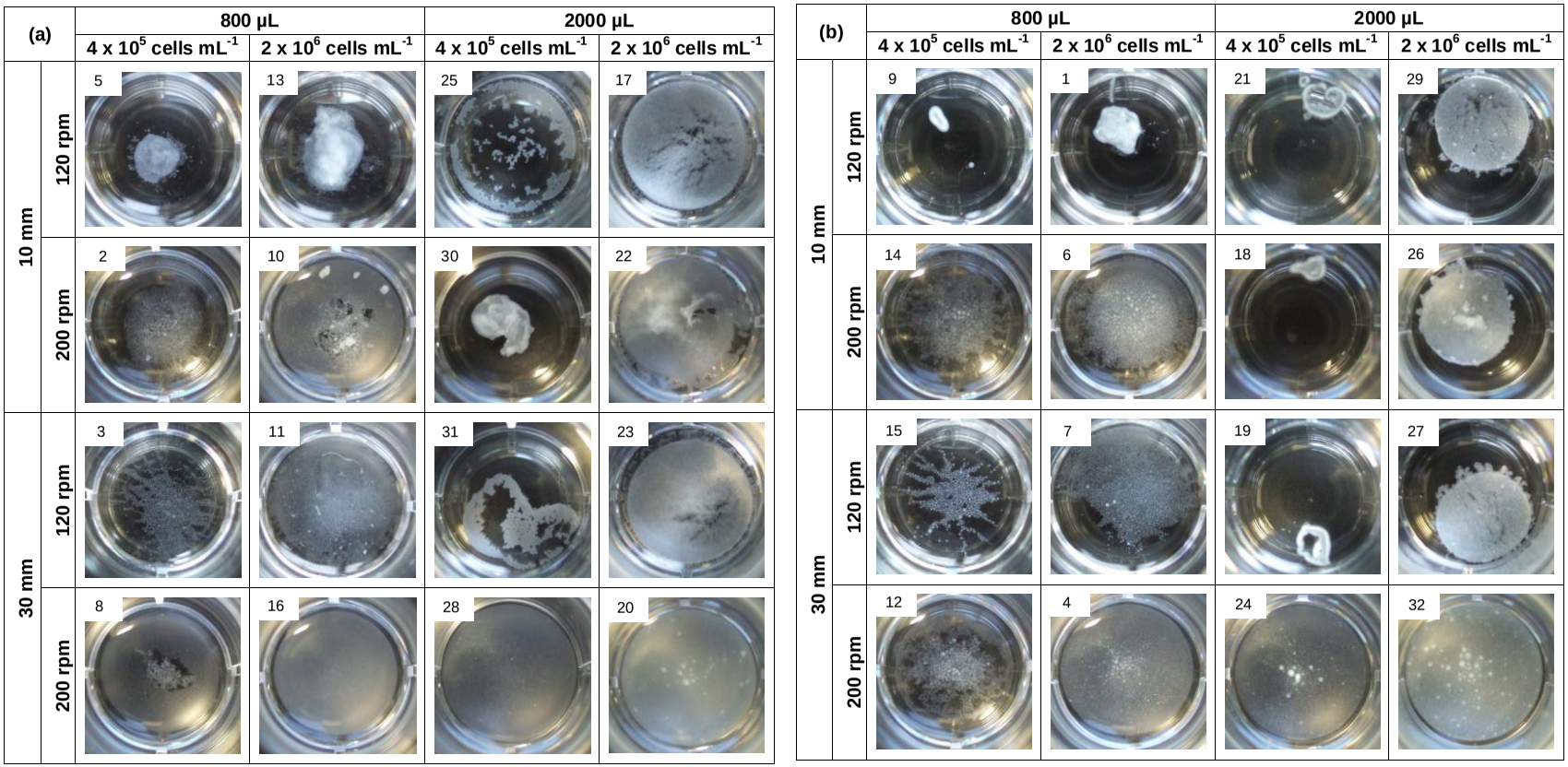


Figure 3.11 Images of PS46.2 suspension cells grown in microwells in medium containing (a) 1 % FCS or (b) 5 % FCS. Cultures were not induced and photographs were taken 46 hr subsequent to seeding. Column labels denote the liquid fill volume (µL) and cell seeding density (viable cells mL^{-1}), while row labels denote the shaking diameter (mm) and shaking speed (rpm). Each set of operating conditions was evaluated in triplicate, and the image displayed is a representative example of the cell growth observed**.** Numbers ascribed to individual images indicate the experimental run (Table 3.3) best represented by the image, where the influences of pre-induction period (x_3), post-induction period (x_4), concentration of dox (x_8) and concentration of NaBu (x_9) have been disregarded.

Firstly, different hydrodynamic conditions gave rise to distinct aggregate formations. A combination of high shaking speed (200 rpm) and high shaking diameter (30 mm) seemed to favour the formation of cell layer that extended across the base of microwell (Figure 3.11 [a] and [b], bottom row of photographs). A high liquid fill volume (2000 µL) when combined with a high cell density (2 x 10^6 viable cells mL^{-1}) had a similar effect, although the layer of cells did not extend to the edge of the microwell in all instances (Figure 3.11 [a] and [b], right hand column of photographs). For all other microwell combinations, the morphology of cell aggregates was much more diverse. The effect of fluid hydrodynamics on the formation of HEK293 cell aggregates in spinner flasks has been previously discussed. Liu *et al.* (2006) found that both low (25 rpm) and high (100 rpm) impeller speeds favoured the formation of large aggregates that had an average diameter of 351 µm and 442 µm, respectively, following a 7 day culture period. It is hypothesised that this may have been caused by the low fluid velocity at 25 rpm, and by the high incidence of aggregate-aggregate collisions at 100 rpm (Liu *et al.*, 2006). When a mid-range impeller speed of 50 or 75 rpm was used, aggregates were smaller (average diameter 210 µm or 175 µm, respectively, after 7 days culture), and the growth and viability of cultures was improved (Liu *et al.*, 2006). Zhao *et al.* (2007) also reported that the optimum impeller speed in spinner flasks for minimising HEK293 aggregation, while retaining cell growth and viability, was 50 rpm. From the purely pictorial data available, it is difficult to draw firm conclusions about the relationship between fluid hydrodynamics and cellular aggregation in the current study, and such attempts are further complicated by the fact that several factors (not all of them relating to the fluid environment) are at play. It is worth noting, however, that the runs found to yield the highest titres during the screening experiment (runs 10, 11, 14 and 15) all had a low fill volume and high-low or low-high combination of shaking speed and shaking diameter. The data presented in Figure 3.11 indicates that cells likely spread out as a layer on the base of the microwell (rather than combined as a single large mass) during these runs. The low fill volume likely ensured sufficient oxygenation of the cultures (as discussed in Section 3.2.5.1) and, although far from conclusive, the observation that none these runs combined low-low or high-high settings of shaking speed and diameter supports the notion that a mid-range level of agitation favours higher cell productivity.

Secondly, and somewhat predictably, a high cell seeding density (2 x 10^6 viable cells mL^{-1}) tended to increase the size and/or extent of cell aggregates, as compared to a low seeding density of 4 x 10^5 viable cells mL^{-1} (compare adjacent columns in Figure 3.11 [a] and [b]). For HEK293 cells cultured in spinner flasks, it has been previously reported that aggregate diameter increased over time as the cell population expanded (Liu *et al.*, 2006; Liu *et al.*, 2009). This is probably because cell-cell collisions, from which aggregate formation may arise, become more likely as cell densities increase. Liquid fill volume was also found to affect the arrangement of cellular aggregates, with differing results depending on the particular shaking conditions and serum concentration employed (compare alternate columns in Figure 3.11 [a] and [b]). It should be acknowledged that for a given starting cell density (no. of viable cells mL^{-1}), the *total* number of cells per well was 2.5 times greater when a fill volume of 2000 µL, as compared to 800 µL, was used. Fill volume therefore not only influenced the mixing characteristics of the culture, but also affected the total number of cells present, and both these factors may have played a role in determining the arrangement of cellular aggregates.

Finally, a high concentration of FCS in the medium (5 %) led to the formation of aggregates that were more closely compacted, as compared to when a low concentration of FCS (1 %) was used (compare photographs in Figure 3.11 [a] with their counterpart in Figure 3.11 [b]). It is probable that the calcium component of the FCS was responsible for this effect. Peshwa *et al.* (1993) previously reported that suspension cultured HEK293 cells rapidly formed clumps upon transferral from a medium containing 0.1 mmol L^{-1} calcium ions (Ca^{2+}) to one containing 0.5 mmol L^{-1} Ca^{2+}, while cell growth rates were unaffected. Zhao *et al.* (2007) reported a positive linear relationship between Ca^{2+} concentration (0.0 - 0.4 mmol L^{-1}) and the diameter of HEK293 cell aggregates, which varied in size from approximately 25 to 65 µm (quantified 36 hr post-seeding). Throughout the feasibility studies described in Sections 3.2.1 and 3.2.3, Ca^{2+} concentration data was collected (Ca^{2+} concentration and pH were measured simultaneously using the blood gas analyser). This data revealed that the Ca^{2+} concentration was lower for HEK293T cultures supplemented with 1 % FCS (0.27 ± 0.00 mmol L^{-1} [mean ± SD]; n = 6) than for HEK293T-derived (PS46.2) cultures supplemented with 5 % FCS (0.40 ± < 0.01 mmol L^{-1} [mean ± SD]; n = 6) when measured immediately post-seeding. Thus, higher Ca^{2+} concentrations accompany higher FCS concentrations, and the supposition that increased

levels of Ca^{2+} are responsible for the denser aggregation observed in the current experiment is therefore plausible.

In general, high levels of aggregation are undesired as cells in the centre of clumps can become non-viable due to diffusional limitations (Peshwa *et al.*, 1993). Also, with regard to lentiviral vector production, diffusional limitations may impede the induction of cells in the centre of clumps and the close crowding of cells may obstruct viral budding, which together would compromise the overall productivity of the culture. The extreme aggregative behaviour observed during the screening experiment may have negatively impacted on titres which were, on the whole, low (Table 3.3). The data presented here should inform the design of microwell-based bioprocess development studies – operating conditions producing aberrant aggregate morphologies that are unlikely to either: (i) yield optimal titres or (ii) be replicable upon scale-up, should ideally be avoided.

3.3 Chapter discussion

3.3.1 Establishment of a microwell platform for use in lentiviral vector process development

The first objective of this chapter was to establish whether a shaken 24-well plate system could support suspension cultures of lentiviral vector producer cell lines. A HEK293T-derived producer cell line (PS46.2) was seeded in microwells (0.8 mL working volume) and culture kinetics and lentiviral vector (ProSavin®) production were assessed. Data was compared to that obtained from parallel shake flask cultures (50 mL working volume), which represent the current standard small-scale culture vessel, and for which operating protocols had been previously established. Overall, the culture kinetics between the two vessel types (in terms of cell growth, pH, and metabolite consumption and production) were similar, and lentiviral vector (ProSavin®) titres were also comparable (Sections 3.2.1 and 3.2.2). Although some degree of evaporation was expected from microwells, the effect on culture osmolalities was slight and should not inhibit use of this platform for batch cultivations. Together, the data indicate that microwells could be used effectively in place of shakes flasks for early phase process development studies based on the PS46.2 cell line, greatly increasing the opportunity for parallelisation and thus experimental throughput. To date, microscale methods for mammalian cell culture have

primarily been employed to aid the development of recombinant protein production processes (Girard *et al.*, 2001; Davies *et al.*, 2005; Micheletti *et al.*, 2006; Barrett *et al.*, 2010; Silk *et al.*, 2010), and this is the first study, to my knowledge, to employ microwells with a view to optimising the manufacturing process for a viral vector.

The microwell operating conditions employed in this study were also found to support successful culture of another cell line (HEK293T not stably transfected with lentiviral vector DNA) grown in medium containing 1 % FCS. A similar degree of comparability between microwell and shake flask cultures was demonstrated as in the PS46.2 study, with regards to cell growth, pH, osmolality and metabolite concentrations (Section 3.2.3). This demonstrates that the applicability of the microwell platform may be extended to other HEK293T cell derivatives.

This work also facilitated insight into the metabolism of key nutrients by the PS46.2 cell line, the findings of which could later inform process design, particularly if extended cultivations were desired. A lack of glutamine and an accumulation of toxic levels of ammonia during the latter stages of the culture were likely limiting factors, while glucose availability and lactate build-up (leading to a lowering of pH) were of lesser concern. Of interest was the observation that the HEK293T cell line responded differently to lactate accumulation than PS46.2, indicating that conclusions specifically relating to one cell line should not be generalised, even to other similar cell types. Results obtained at the microscale can therefore be used to characterise differences in cell lines and their metabolic profile. This can be an important step in cell line selection and in defining ultimate scale-up requirements.

3.3.2 Rejection of mixing time as a potential scaling criterion

As it was critical that data generated using the microwell platform remained relevant upon scale-up, the second objective of this chapter was to identify an appropriate criterion on which to base scale translation studies. As scale-up from microwells to WAVE bioreactors had been previously achieved on the basis of mixing time (Gill *et al.*, 2011), the relevance of this parameter for scale-up of the lentiviral vector production process was evaluated. For a microwell operated under conditions found to yield acceptable cell growth kinetics and lentiviral vector titres, the mixing time (measured using the iodine decolourisation method) was extremely

long (1037 s), and could not be matched in a WAVE bioreactor (for example, mixing times for a 2 L system were reported to range from 9 to 264 s (Eibl *et al.*, 2010b)) (Section 3.2.4). Consequently, the use of mixing time as a scaling criterion was deemed impracticable and alternative criteria were sought using a DoE methodology.

3.3.3 Identification of three factors that significantly impact on ProSavin® titres

The third objective of this chapter was to demonstrate the utility of the microwell platform for the rapid, early generation of relevant bioprocess design data. A microwell-based DoE-guided approach to bioprocess development was initiated, the first stage of which was experimental screening. The relative influence of nine factors on ProSavin® titres was assessed. Each factor was investigated over two levels using a fractional factorial design. Three factors (post-induction period, liquid fill volume and concentration of dox) were found to have a significant impact on titre (Section 3.2.5.1). By identifying those factors which most impacted on ProSavin® titres, the screening experiment achieved its primary aim. It is clear that the sensitivity of titres to changes in post-induction period, liquid fill volume, and concentration of dox should be given particular consideration when designing a manufacturing route for lentiviral vectors, and these factors were thus taken forward for further optimisation studies (as described in Chapter 4). It should also be noted, however, that although the other six parameters were found not to significantly impact on ProSavin® titres in the present work, these results should be viewed in light of the operational ranges over which each parameter was investigated (Table 3.2), i.e. if broader ranges had been chosen, a greater impact on titres may have been observed. It was noted, however, that these factors played a role in determining the size and arrangement of PS46.2 aggregates. Increasing the cell density or concentration of FCS tended to increase aggregation, while the relationship between the fluid hydrodynamics (which was dependent on the shaking speed, shaking diameter, and liquid fill volume) and aggregation was more difficult to define (Section 3.2.5.2). Cellular aggregation is undesirable as it may reduce titres and compromise scale-up studies, thus it is valuable to develop an understanding of how various operating parameters can contribute to this phenomenon. This study represents the first application of a DoE approach to lentiviral vector upstream development. As the use of suspension-adapted stable producer cell lines is highly desirable for future large scale

productions of clinical grade lentiviral vectors, the insights gained here should aid researchers working in this field.

4 Characterisation of lentiviral vector production using the microwell platform

4.1 Introduction and aims

Existing manufacturing processes for lentiviral vectors, based on the transient transfection of adherent monolayers of HEK293-derived cells (Slepushkin *et al.*, 2003; Negré *et al.*, 2008; Schweizer and Merten, 2010; Merten *et al.*, 2011; Stewart *et al.*, 2011; Ausubel *et al.*, 2012), are not scalable, and for commercialisation the development of stable producer cell lines capable of growth in suspension is desired (Ansorge *et al.*, 2010; Schweizer and Merten, 2010; Segura *et al.*, 2013). However, the propensity of HEK293-derived cells to aggregate when cultured in suspension (Merten *et al.*, 2001), changes in the morphology and membrane properties of producer cells following suspension adaption (Ansorge *et al.*, 2010) and a limited understanding of the culture conditions required for optimal productivity (Segura *et al.*, 2013) has greatly hindered progress in this area and, to date, just one report documenting the successful creation of suspension-grown lentiviral vector producer cell lines has been published (Broussau *et al.*, 2008). In order for scalable manufacturing processes for lentiviral vectors to become a reality, a deeper understanding of the factors that impact on the productivity of suspension-adapted stable producer cells is clearly required.

To address this issue, the overarching aim of this thesis was to test an approach to lentiviral vector process development that was based on a combination of microscale bioprocessing and Design of Experiments (DoE) techniques, which would theoretically allow the effect of multiple operating parameters to be quickly characterised through a series of logically linked experiments (see Sections 1.5.1 and 1.5.2). To help develop and demonstrate the approach, the suspension-adapted tet-ON inducible stable producer cell line, PS46.2, was employed (Stevenson *et al.*, 2009; Stewart *et al.*, 2009; Stewart *et al.*, 2011). Suspension-adapted PS46.2 currently generates lentivirus (ProSavin®) titres that are 5 to 20-fold lower than those required for clinical purposes (see Section 1.4.5). As lack of a scalable bioprocess for ProSavin® could impede its future commercialisation it is important this issue is addressed.

In Chapter 3, a microwell experimental platform was established for use in lentiviral vector process development studies based on suspension cultures of HEK293T-derived producer cells. A DoE screening experiment was subsequently carried out using the microwell platform to identify which operating parameters most significantly impacted on ProSavin® titres from suspension cultures of PS46.2. Out of nine parameters, three were identified as having a substantial influence on ProSavin® titres, which were: post-induction period, liquid fill volume and concentration of dox (Section 3.2.5). The aim of this second results chapter is to model the effects of these important factors on ProSavin® production, with a view to optimising production conditions and informing future scale-up studies. The specific objectives are:

- To employ the microwell platform and DoE methodology to (i) narrow the operating ranges for post-induction period, liquid fill volume and concentration of dox and (ii) subsequently predict their optimum settings.
- To conduct microwell experiments to verify the predictive capability of the final model.
- To describe the fluid mixing within microwells (operating under optimised conditions) using dimensionless numbers, with a view to informing scale translation studies.
- To evaluate whether daily additions of dox to microwell suspension cultures of PS46.2 could boost titres during long (70 hr) induction periods (this objective stemmed from the results associated with first objective).

Results included in this chapter were published in: Guy, H. M., *et al*. (2013). *Human Gene Therapy Methods*, **24(2)**: 125-139. Permission to reproduce this content has been granted by Mary Ann Liebert, Inc.

4.2 Results

4.2.1 Optimisation of ProSavin® titres using microwell cultures of PS46.2 and Design of Experiments (DoE) techniques

4.2.1.1 Overview of the DoE approach employed for optimisation studies

A series of DoE-guided microwell-based experiments were carried out to establish optimal operating conditions for the production of ProSavin® from suspension cultures of PS46.2. This

section provides an overview of the approach employed, including a description of central composite designs.

Of the nine factors initially investigated (Section 3.2.5), three were found to have a critical influence on ProSavin® titre: post-induction period, liquid fill volume and concentration of dox. These factors were thus taken forward for further characterisation and optimisation studies, while all other factors were given a fixed value. Following a broad screening experiment, the impact of a small number of critical factors can be investigated in greater detail using experiments of central composite design. Central composite designs consist of (i) a basic two-level factorial design (i.e. a high and low value for each factor), (ii) repeated centre point experiments for estimation of pure error, and (iii) additional symmetrically arrayed axial points (Anderson and Whitcomb, 2007; Eriksson *et al.*, 2008). The inclusion of axial points means that a quadratic equation can be fitted to response data, enabling curvature in the experimental system to be modelled and subsequently visualised using response surface methodology (Anderson and Whitcomb, 2007; Eriksson *et al.*, 2008). The primary benefit of this approach is that it can direct researchers quickly towards the optimal operating conditions for a given system. In central composite face-centred (CCF) designs, the axial points are at the centre of each face of the factorial design space. In central composite circumscribed (CCC) designs, the axial points are outside of the factorial design space and, in order to retain the symmetry of the design region, are typically situated on the circumference of a circle with radius 1.4 (Eriksson *et al.*, 2008). Consequently, CCF designs require each factor to be varied over three levels (low, medium and high), while CCC designs require each factor to be varied over five levels (low^{a}, low, medium, high, high^{a}), where 'a' denotes that the design point is both axial and outside of the factorial design space.

While CCF designs can be a more practical choice when it comes to data collection (e.g. vector harvests at three different time points is less laborious than vector harvests at five separate time points), a CCC design is better able to capture strong curvature within an experimental system (Eriksson *et al.*, 2008). Here, since the first two experiments described below (Sections 4.2.1.2 and 4.2.1.3) were primarily conducted to identify appropriate ranges for each of the factors a CCF design was deemed sufficient. As the final DoE experiment (Section 4.2.1.4) was conducted to ascertain whether a unique optimum set of conditions existed where maximum

titres could be achieved, a CCC design was considered most appropriate. For statistical calculations the levels of each variable were coded as described previously (see Section 2.4.1.1 and Islam *et al.* (2007)), while axial points included in the CCC design were coded as +/- 1.4.

4.2.1.2 A central composite face-centred (CCF) experiment to locate the optimal operating ranges for post-induction period, liquid fill volume and concentration of dox

Following on from the screening experiment described in Section 3.2.5 the next stage was to narrow the operating ranges for those factors found to significantly impact on ProSavin® titres. As each factor had been previously investigated over just two levels, it was unknown whether the optimal settings for these factors had been included within the experimental region investigated during screening. Thus this follow-on study was conducted with the experimental objective to locate the optimal region (see Section 1.5.2). Post-induction period, liquid fill volume and dox concentration were varied over the ranges 22 - 70 hr, 600 - 1000 µL and 1.0 - 2.6 µg mL^{-1}, respectively, using a CCF design, which enabled the impact of each factor to be examined over three levels (Section 4.2.1.1). The high and low settings for each factor were chosen in accordance with the results obtained from the screening experiment, such that each factor range was varied symmetrically around the previous optimum identified. The remaining six factors, that had not by themselves exerted a significant influence on ProSavin® titres during experimental screening, were given a fixed value. As it was conceivable that the following pairs of factors: (i) shaking speed and shaking diameter, and (ii) pre-induction period and cell seeding density, may have acted synergistically on ProSavin® titres during the screening experiment it was deemed prudent to fix these factors at midpoint values for optimisation studies, avoiding extreme combinations (e.g. high shaking speed plus high shaking diameter). The serum concentration of the media, and the concentration of NaBu (added at induction) were fixed at their high and low values (Table 3.2), respectively, as these were the values used during the experimental run that yielded the highest titre during screening (run 14, Table 3.3). The materials and methods employed in this study are detailed in Section 2.4.2, and the settings for all factors are summarised in Table 4.1. To increase our understanding of the impact of the chosen variable factors, the number of responses measured was also increased. ProSavin® titre (functional vector) measurements were supplemented with RNA copy number measurements to give an indication of total viral particle numbers. Viable cell concentration and

cell viability at the time of harvest was also quantified. The design matrix and results for this experiment are presented in Table 4.2. ProSavin® titre and particle:infectivity (P:I) ratio (RNA copy number / titre) values were entered into DoE software so that the behaviour of the system could be modelled.

Table 4.1 Specification of the factors and settings investigated during the first central composite face-centred (CCF) experiment. Post-induction period, liquid fill volume and concentration of dox (doxycycline) were investigated over the ranges specified, while the other six factors were given a fixed value.

Factor (units)	Abbreviation	Fixed value	Settings		
			-1	0	1
Shaking speed (rpm)	x_1	160	-	-	-
Shaking diameter (mm)	x_2	20	-	-	-
Pre-induction period (hr)	x_3	24	-	-	-
Post-induction period (hr)	***x_4***	***-***	***22***	***46***	***70***
Liquid fill volume (µL)	***x_5***	***-***	***600***	***800***	***1000***
Cell seeding density (viable cells mL^{-1})	x_6	1.2×10^6	-	-	-
Concentration of serum (% [v/v])	x_7	5	-	-	-
Concentration of dox (µg mL^{-1})	***x_8***	***-***	***1.0***	***1.8***	***2.6***
Concentration of NaBu (mM)	x_9	2	-	-	-

Table 4.2 CCF design matrix for investigation into the effect of post-induction period (x_4), liquid fill volume (x_5) and concentration of dox (x_8) on cell growth and lentiviral vector production. Each factor was varied over three levels and six centre point experiments were included for estimation of pure error. Factors and their ranges were coded according to Table 4.1. Each run was performed in triplicate and, where relevant, mean values ± 1 SD are presented. Cell concentration and viability measurements were performed immediately prior to lentiviral vector harvests. P:I ratio is an abbreviation of particle:infectivity ratio, which was calculated using the mean values for RNA copy number and titre (P:I ratio = RNA copy number / titre). Predicted values were calculated from the regression model fitted to a given response (see Section 2.4.2), and were computed using the "point prediction" tool within DoE software (similar predicted and measured values are indicative of a well-fitting model).

Run	x_4	x_5	x_8	Viable cells mL^{-1} (x 10^6)	Cell viability (%)	RNA copies mL^{-1} (x 10^9)	Titre (x 10^4 TU mL^{-1})		P:I ratio (x 10^5)	
							Measured	Predicted	Measured	Predicted
1	-1	-1	-1	2.4 ± 0.7	92.6 ± 0.5	7.3 ± 1.3	3.2 ± 0.3	3.3	2.3	2.2
2	1	-1	-1	4.4 ± 0.2	73.1 ± 1.0	17.5 ± 5.0	1.2 ± 0.1	1.2	14.5	15.4
3	-1	1	-1	2.5 ± 0.3	91.4 ± 3.5	5.4 ± 0.7	3.7 ± 0.5	3.7	1.5	1.5
4	1	1	-1	2.6 ± 0.4	58.0 ± 3.0	13.5 ± 3.3	1.2 ± 0.1	1.2	11.1	10.5
5	-1	-1	1	3.5 ± 0.7	94.1 ± 1.7	5.7 ± 1.4	3.4 ± 0.4	3.5	1.7	1.6
6	1	-1	1	4.3 ± 0.1	74.8 ± 1.4	13.3 ± 3.3	1.5 ± 0.3	1.5	8.9	9.2
7	-1	1	1	2.3 ± 0.6	88.5 ± 1.6	5.9 ± 2.0	4.0 ± 0.4	4.0	1.5	1.4
8	1	1	1	2.1 ± 0.9	61.0 ± 7.2	13.4 ± 1.9	1.6 ± 0.2	1.5	8.6	8.3
9	-1	0	0	2.4 ± 0.3	91.5 ± 1.2	6.6 ± 0.8	3.9 ± 0.4	3.6	1.7	1.7
10	1	0	0	2.2 ± 0.7	58.3 ± 5.7	15.3 ± 4.6	1.3 ± 0.2	1.3	12.1	10.5
11	0	-1	0	4.1 ± 0.5	86.4 ± 1.2	12.7 ± 0.6	2.5 ± 0.2	2.9	5.0	4.7
12	0	1	0	2.5 ± 0.2	80.2 ± 4.9	10.3 ± 0.7	2.9 ± 0.3	3.2	3.5	3.7
13	0	0	-1	3.2 ± 0.4	81.8 ± 2.1	12.1 ± 3.9	2.6 ± 0.2	2.7	4.6	4.8
14	0	0	1	2.6 ± 0.5	78.1 ± 4.0	12.3 ± 2.9	3.5 ± 0.2	3.6	3.5	3.6
15	0	0	0	3.6 ± 0.5	85.3 ± 1.7	13.4 ± 2.2	3.1 ± 0.3	3.0	4.3	4.2
16	0	0	0	2.6 ± 0.3	80.0 ± 2.3	12.0 ± 3.8	2.9 ± 0.1	3.0	4.1	4.2
17	0	0	0	2.4 ± 0.4	78.0 ± 1.2	11.8 ± 3.2	3.1 ± 0.2	3.0	3.8	4.2
18	0	0	0	2.4 ± 0.4	78.5 ± 2.2	12.5 ± 2.4	3.3 ± 0.3	3.0	3.8	4.2
19	0	0	0	2.8 ± 0.2	78.4 ± 1.0	13.0 ± 2.9	3.4 ± 0.4	3.0	3.8	4.2
20	0	0	0	2.2 ± 0.8	79.5 ± 2.8	14.2 ± 2.1	3.4 ± 0.2	3.0	4.2	4.2

A quadratic model was fitted to titre data, which had undergone an inverse transformation. The resulting model yielded an adjR^2 value of 0.98 and predR^2 value of 0.98 following refinement (which was carried out described in Section 2.4.2). As the difference between the adjR^2 and predR^2 was < 0.2 and the predR^2 value was ≥ 0.9, the model fit was deemed excellent. Analysis of variance (ANOVA) statistics revealed that the model was significant (p < 0.0001) and that there was no significant 'lack of fit' (p = 0.1806). Model coefficients for ProSavin® titre responses are presented in Figure 4.1. From the model coefficients, it is evident that post-induction period exerted the largest effect on titre (p < 0.0001). The direction of this effect was negative, indicating that higher titres were achieved when harvests were performed earlier, at 22 hr, as opposed to 70 hr, post-induction. The model term (post-induction period)^2 was also significant (p < 0.0001). As squared terms represent curvature in the model, this indicates that the relationship between post-induction period and titre was non-linear. Further analysis of the data using DoE software revealed that maximum titres were most likely to be obtained sometime between 22 hr and 46 hr post-induction. Dox concentration exerted a smaller effect on titre than post-induction period, and the direction of this effect was positive, indicating that the higher value (2.6 µg mL^{-1}) yielded higher titres (p < 0.0001). However, there was an interaction between these two factors (p = 0.0015), and exploration of the data using DoE software revealed that higher titres were only achieved at the high dox concentration when the post-induction period was prolonged (70 hr). Liquid fill volume did not exert a significant effect on titre over the range studied in this experiment (p = 0.0654). The maximum titre achieved in this first experiment was 4.0 x 10^4 TU mL^{-1}, where the post-induction period was 22 hr, liquid fill volume was 1000 µL and dox concentration was 2.6 µg mL^{-1} (Run 7, Table 4.2).

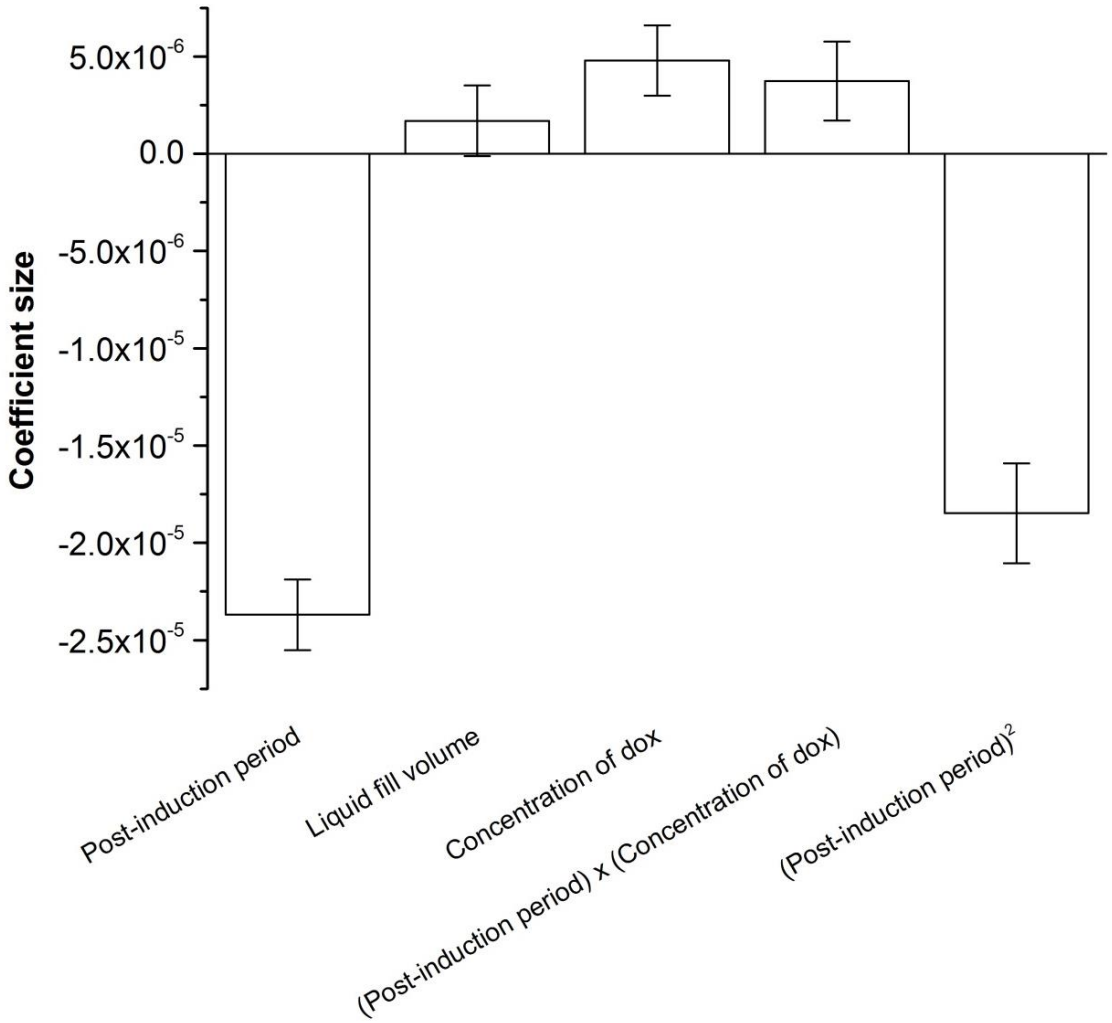


Figure 4.1 Coefficient plot of the refined quadratic model for titre (inverse transformed) with confidence intervals set at 95 %. The size of each coefficient illustrates the relative influence of that factor on titre. A positive coefficient indicates that an increase in that factor (relative to its centre point value) would result in an increase in titre, while the reverse is true for negative coefficients. Due to the inverse transformation of titre data, it was necessary to multiply coefficients by -1 to enable appropriate interpretation (in terms of the direction of the effect). Post-induction period, liquid fill volume and concentration of dox were investigated over the ranges 22 - 70 hr, 600 - 1000 µL and 1.0 - 2.6 µg mL^{-1}, respectively, using a central composite face-centred (CCF) design (Table 4.1). Coefficients with confidence intervals that do not span zero are considered significant at the level $p < 0.5$.

Titre data can be utilised in conjunction with RNA copy number data to calculate the proportion of particles in a given suspension that are functional (the P:I ratio). For the conditions examined in the present experiment, the P:I ratio ranged from 1.6 x 10^5 to 1.5 x 10^6 (Table 4.2). A two-factor interaction model was fitted to P:I ratio values, which had undergone a log transformation.

The resulting model yielded an adjR^2 value of 0.99 and predR^2 value of 0.98 following refinement (which was carried out as described in Section 2.4.2), again indicating excellent model fit. ANOVA statistics revealed that the model was significant ($p < 0.0001$), and that there was no significant 'lack of fit' ($p = 0.2058$). Model coefficients for P:I ratio responses are presented in Figure 4.2. A high P:I ratio is undesirable as this would indicate that the majority of particles within the harvested supernatant are non-functional. Here, post-induction period had the greatest effect ($p < 0.0001$), and early harvests (22 hr) yielded a desirably lower P:I ratio. Using the data in Table 4.2, it may be observed that late harvests (70 hr) were associated with lower cell viabilities, lower titres and higher RNA copy numbers. This may suggest that the increased worsening P:I ratio associated with longer harvest periods was caused by a release of lentiviral vector RNA into the supernatant as a result of cell death. Liquid fill volume ($p < 0.0001$) and concentration of dox ($p < 0.0001$) had a relatively lesser effect on the P:I ratio, but nonetheless it was indicated that at higher settings of these (1000 µL and 2.6 µg mL^{-1}, respectively) a greater proportion of lentiviral vector particles were functional. However, interaction effects existed between post-induction period and concentration of dox ($p = 0.0413$), and between liquid fill volume and concentration of dox ($p = 0.0118$). Further exploration of the data using DoE software revealed that, in fact, neither liquid fill volume or concentration of dox alone affected the P:I ratio, and that the associated interaction effects were complex: a lower P:I ratio was only achieved at (i) the high liquid fill volume (1000 µL) if the concentration of dox was low (1.0 µg mL^{-1}), and (ii) at the high dox concentration (2.6 µg mL^{-1}) if the liquid fill volume was low (600 µL) or, if the fill volume was high (1000 µL), the post-induction period was prolonged (70 hr). Overall, the influence of these factors was small as compared to the impact of post-induction period (Figure 4.2), and it is possible that these minor effects were an artefact of the experimental procedure. For this reason, and because P:I ratio data was of secondary interest after titre data, the minor contributions liquid fill volume and concentration of dox made to the model were not explored further.

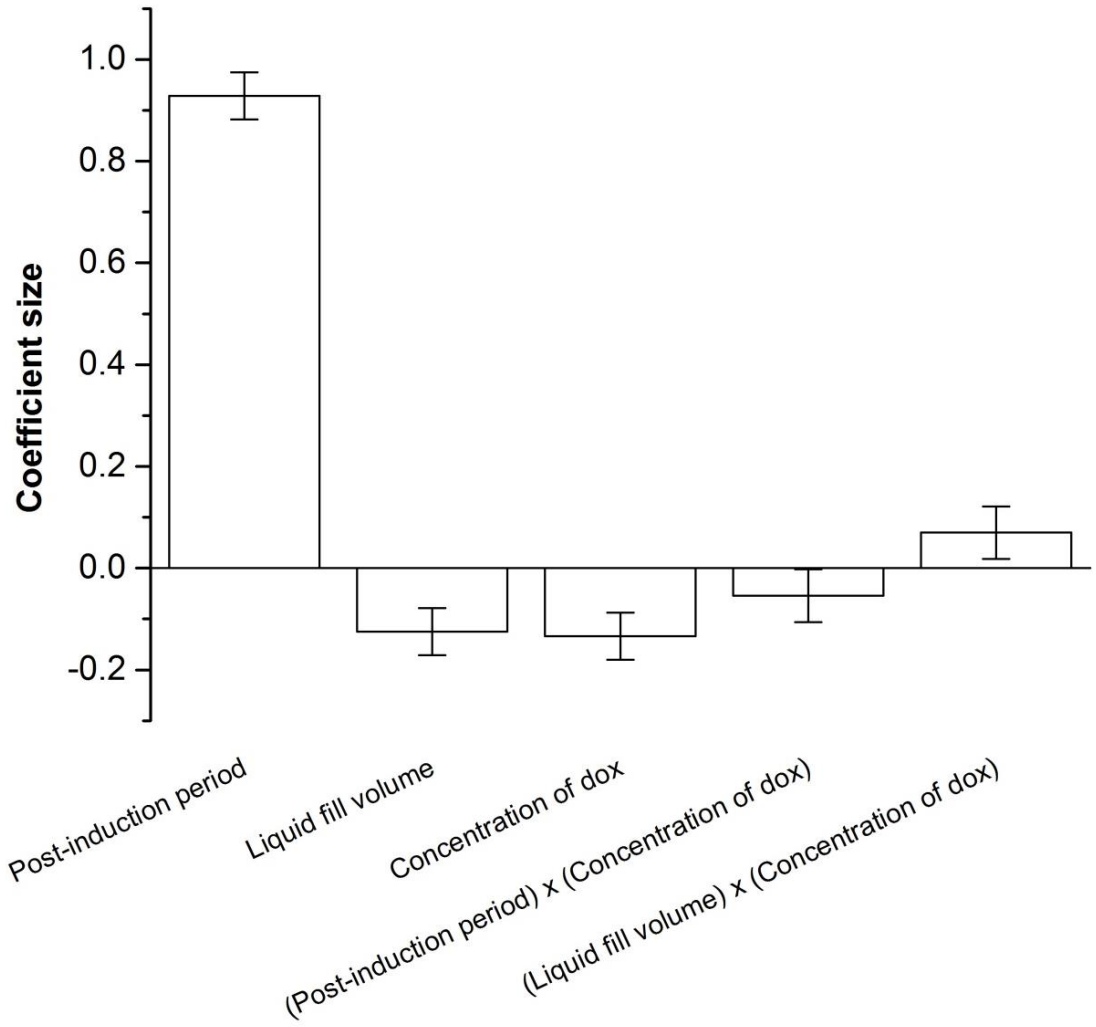


Figure 4.2 Coefficient plot of the refined two-factor interaction model for particle:infectivity (P:I) ratio (log transformed) with confidence intervals set at 95 %. Factors and their ranges, and the experimental design were as noted for Figure 4.1.

To briefly summarise, post-induction period was the factor that most influenced ProSavin® titres and the P:I ratio in this experiment. During experimental screening it was demonstrated that after 6 hr, titres were negligible (Section 3.2.5.1). In this study, moderate titres were obtained between 22 and 70 hr post-induction, and earlier (22 hr) harvests were preferable in terms of maximising titres and minimising the P:I ratio (Table 4.2). When the post-induction period was extended to 70 hr, titres dropped and the P:I ratio worsened (see Figures 4.1 and 4.2), indicating that functional ProSavin® is inactivated in culture supernatants over time, and by 70 hr post-induction the rate of decay exceeds the rate of production. Late harvests (70 hr post-induction) were associated with reduced cell viabilities (< 75 %; Table 4.2), suggesting that cell

death may have contributed to the decline in ProSavin® production. Cell death may have resulted from nutrient limitations, such as a lack of glutamine or an accumulation of ammonia (Section 3.2.1.4), or from a build-up of the lentiviral vector envelope protein, VSV-G - the cytotoxic properties of which have been well documented (Burns *et al.*, 1993; Yee *et al.*, 1994; Yang *et al.*, 1995; Ory *et al.*, 1996). HIV-based lentiviral vectors have been reported to decay rapidly at 37 °C, with a half-life of between 0.8 and 10.4 hr (Higashikawa and Chang, 2001; Carmo *et al.*, 2009a; Carmo *et al.*, 2009b). In order to observe if the responses are a function of the vector half-life itself, the stability of ProSavin®, and other EIAV-derived lentiviral vectors, will be investigated later in Chapter 6. From the regression models generated here, the optimum harvest time (in terms of maximising ProSavin® titres and minimising the P:I ratio) was predicted to fall between 22 and 46 hr post-induction. The liquid fill volume, which was varied between 600 and 1000 µL, did not affect ProSavin® titres. A high dox concentration (2.6 as opposed to 1.0 µg mL^{-1}) yielded slightly higher titres, but only following extended cultivations (70 hr post-induction). Therefore this improvement could not be considered to be of real benefit, as harvests performed at 70 hr post-induction still yielded poorer titres than the earlier harvests performed at 22 hr post-induction. A secondary experiment was conducted to confirm that the amount of residual dox left over from cell inductions did not affect the transduction stage of the ProSavin® titre assay. ProSavin®-containing supernatants that had been harvested from standard shake flask cultures (methods as described in Section 2.4.1.3) were spiked with 0.0, 1.3 or 2.6 µg mL^{-1} dox (supplementary to the 1.0 µg mL^{-1} which had been added during normal inductions) prior to storage at -80°C. ProSavin® titres were then quantified using the rapid flow cytometry method described in Section 2.11.2.1. As illustrated in Figure 4.3, the addition of extra dox to supernatants prior to performing cell transductions had no effect on ProSavin® titre, thus the presence of varying amounts of residual dox in harvested supernatants should not influence the efficiency of cell transductions performed during the titre assay.

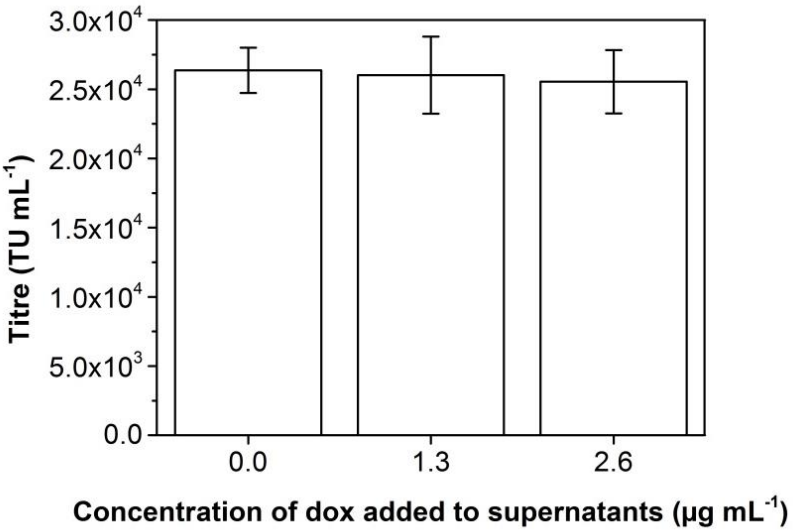


Figure 4.3 The influence of dox on cell transductions. ProSavin®-containing supernatants that had been harvested from standard shake flask cultures (Section 2.4.1.3) were spiked with varying concentrations of dox prior to storage at -80°C, following which titres were quantified according to standard protocol (Section 2.11.2.1). Mean values are presented, and error bars represent ± 1 SD (n = 3).

4.2.1.3 A CCF experiment to further refine the operating ranges for post-induction period and concentration of dox

Prior to conducting the final optimisation experiment (from which optimal factor settings might be predicted), it was necessary to further refine the experimental ranges for post-induction period and concentration of dox. In terms of post-induction period, it was desirable to establish whether optimal titres were likely to be achieved between 6 and 22 hr post-induction, or whether this period could be excluded from the final experiment – enabling a narrower experimental region to be investigated in greater detail. With regards to dox concentration, it seemed prudent to evaluate whether increasing the dox concentration beyond 2.6 µg mL^{-1} could further increase ProSavin® titres during early harvests. Post-induction period and concentration of dox were therefore varied over the ranges 18 - 26 hr and 1.0 - 5.0 µg mL^{-1}, respectively, using a CCF design, while all other factors were given a fixed value (Table 4.3). Liquid fill volume was fixed at 1000 µL, as during the previous CCF experiment (Section 4.2.1.2), runs conducted using a 1000 µL liquid fill volume yielded slightly higher titres than those conducted using a 600 µL fill

volume (although this effect was not statistically significant: p = 0.0654). The materials and methods employed in this study are detailed in Section 2.4.3. The same responses were measured as for the previous CCF experiment, and the design matrix and results are presented in Table 4.4.

Table 4.3 Specification of the factors and settings investigated during the second CCF experiment. Post-induction period and concentration of dox were investigated over the ranges specified, while the other seven factors were given a fixed value.

Factor (units)	Abbreviation	Fixed value	Settings		
			-1	0	1
Shaking speed (rpm)	x_1	160	-	-	-
Shaking diameter (mm)	x_2	20	-	-	-
Pre-induction period (hr)	x_3	24	-	-	-
Post-induction period (hr)	***x_4***	***-***	***18***	***22***	***26***
Liquid fill volume (µL)	x_5	1000	-	-	-
Cell seeding density (viable cells mL^{-1})	x_6	1.2×10^6	-	-	-
Concentration of serum (% [v/v])	x_7	5	-	-	-
Concentration of dox (µg mL^{-1})	***x_8***	***-***	***1.0***	***3.0***	***5.0***
Concentration of NaBu (mM)	x_9	2	-	-	-

Table 4.4 CCF design matrix for investigation into the effect of post-induction period (x_4) and concentration of dox (x_8) on cell growth and lentiviral vector production. Each factor was varied over three levels and six centre point experiments were included for estimation of pure error. Factors and their ranges were coded according to Table 4.3. Each run was performed in triplicate and, where relevant, mean values ± 1 SD are presented. Cell concentration and viability measurements were performed immediately prior to lentiviral vector harvests. Predicted values were calculated from the regression model fitted to titre data (see Section 2.4.3), and were computed using the "point prediction" tool within DoE software (similar predicted and measured values are indicative of a well-fitting model).

Run	x_4	x_8	Viable cells mL^{-1} ($x 10^6$)	Cell viability (%)	RNA copies mL^{-1} ($x 10^9$)	Titre ($x 10^4$ TU mL^{-1})		P:I ratio ($x 10^5$)
						Measured	Predicted	
1	-1	-1	4.2 ± 0.7	96.7 ± 0.5	3.5 ± 0.6	3.5 ± 0.1	3.6	1.0
2	1	-1	4.1 ± 0.3	97.3 ± 0.3	4.7 ± 1.4	6.2 ± 1.1	5.9	0.8
3	-1	1	4.0 ± 0.2	93.9 ± 1.8	3.2 ± 0.7	3.4 ± 0.5	3.6	0.9
4	1	1	3.7 ± 0.4	96.8 ± 0.6	6.1 ± 0.8	5.9 ± 0.3	5.9	1.0
5	-1	0	3.0 ± 0.3	93.5 ± 0.6	3.0 ± 0.9	3.9 ± 0.1	3.6	0.8
6	1	0	3.3 ± 0.4	96.0 ± 1.2	5.4 ± 1.0	5.5 ± 0.4	5.9	1.0
7	0	-1	3.7 ± 0.7	96.8 ± 0.1	3.6 ± 0.5	3.5 ± 0.8	3.3	1.0
8	0	1	3.0 ± 0.2	96.6 ± 0.3	3.8 ± 0.7	3.2 ± 0.4	3.3	1.2
9	0	0	2.6 ± 0.2	95.8 ± 0.2	3.7 ± 0.5	3.4 ± 0.9	3.3	1.1
10	0	0	2.7 ± 0.1	96.5 ± 0.2	4.2 ± 0.5	3.8 ± 0.2	3.3	1.1
11	0	0	2.4 ± 0.1	94.7 ± 0.3	4.1 ± 0.8	4.1 ± 1.5	3.3	1.0
12	0	0	2.8 ± 0.4	96.0 ± 0.6	3.3 ± 0.8	3.0 ± 0.2	3.3	1.1
13	0	0	3.1 ± 0.8	97.3 ± 0.8	3.0 ± 0.8	2.7 ± 0.7	3.3	1.1
14	0	0	2.6 ± 0.2	95.8 ± 1.4	2.9 ± 1.2	3.0 ± 0.2	3.3	1.0

A quadratic model was fitted to titre data, and the resulting model yielded an adjR^2 value of 0.86 and predR^2 value of 0.82 following refinement (which was carried out as described in Section 2.4.3). The model fit could therefore be considered good. ANOVA statistics revealed that the model was significant ($p < 0.0001$), and that there was no significant 'lack of fit' ($p = 0.9524$). Only post-induction period and (post-induction period)^2 were included as significant model terms ($p < 0.0001$). Figure 4.4 has been included to illustrate the impact of post-induction period and dox concentration on titre and RNA copy number data. 'Late' harvests (26 hr post-induction) yielded the highest titres and, interestingly, from 22 to 26 hr a 67 to 84 % increase in mean titre was observed (Table 4.4; Figure 4.4), suggesting that even a few hours difference in harvest timing can be critical. From this data it may be concluded that 22 hr would be an appropriate lower threshold for post-induction period in the final optimisation experiment. Varying the dox

concentration did not affect ProSavin® titres in this experiment, indicating that 1.0 µg mL^{-1} was sufficient for full cell induction over this timeframe. The maximum titre achieved in this second CCF experiment was 6.2 x 10^4 TU mL^{-1}, where the post-induction period was 26 hr and the dox concentration was 1.0 µg mL^{-1} (Run 2, Table 4.4). It may be observed that RNA copy number values rose in line with increasing titres (Figure 4.4), thus the P:I ratio remained relatively consistent over time (ranging from 0.8 to 1.2 x 10^5; Table 4.4). Exploration of the P:I ratio data using DoE software revealed that neither factor significantly impacted on this response. Cell viabilities were high (> 93 %) for all runs, while cell concentrations ranged between 2.4 and 4.2 x 10^6 viable cells mL^{-1} (Table 4.4).

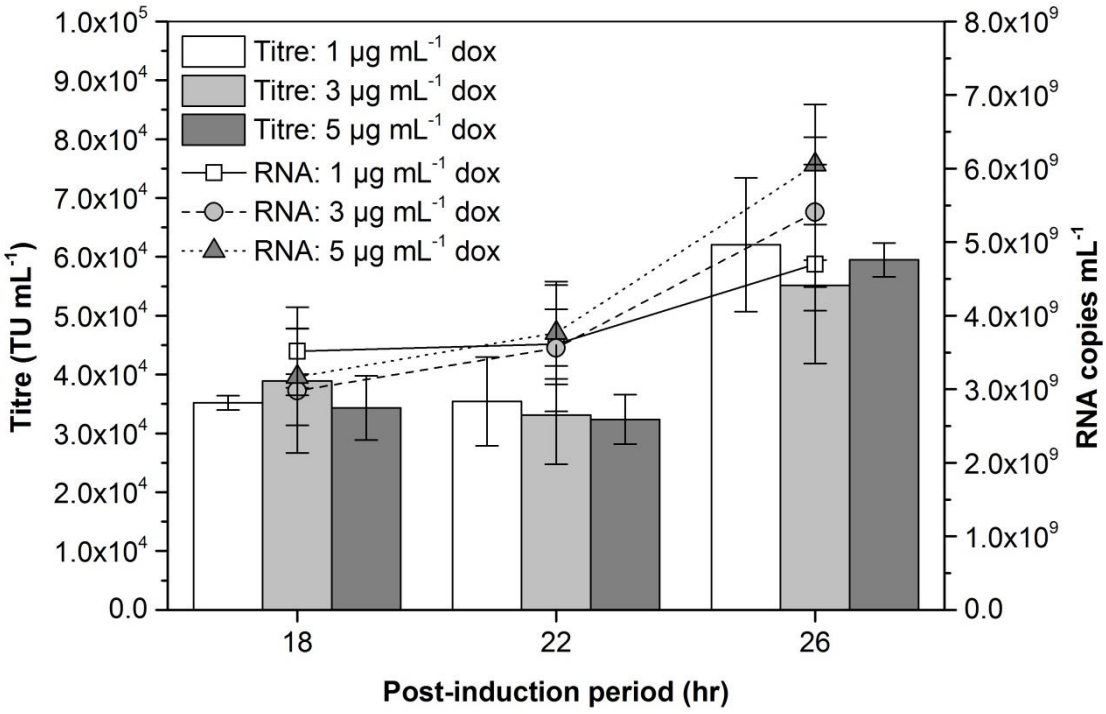


Figure 4.4 Effect of post-induction period and concentration of dox on titre (bars) and RNA copy number (lines). Mean values are presented, and error bars represent ± 1 SD (n ≥ 3). All other operating parameters were fixed as outlined in Table 4.3.

4.2.1.4 A central composite circumscribed (CCC) experiment to predict the optimum values for post-induction period and liquid fill volume

A final DoE experiment was conducted to predict the optimum values for post-induction period and liquid fill volume. Experimental screening had identified these factors, along with dox concentration, as significantly impacting on ProSavin® titres (Section 3.2.5). Subsequent experiments to narrow the operating ranges for these factors had demonstrated that: (i) the optimal setting for post-induction period was likely to fall between 22 and 46 hr, (ii) 1.0 µg mL^{-1} dox appeared sufficient for cell induction when harvests were performed after approximately 22 hr, however for later harvests (70 hr) a higher dox concentration (2.6 µg mL^{-1}) conferred a slight benefit, and (iii) titre was not affected by liquid fill volume over the range 600 to 1000 µL (Sections 4.2.1.2 and 4.2.1.3). In this experiment, post-induction period was investigated over the range 22 - 46 hr, as this was expected to include the optimum setting. Liquid fill volume was examined over the range 700 - 1300 µL. This range was chosen as, although a 2000 µL fill volume was previously found to negatively impact on titres (Section 3.2.5), the region between 1000 and 2000 µL had not been previously explored. Although in the first CCF experiment liquid fill volume did not have a significant impact on titre over the range 600 - 1000 µL (p = 0.0654), the p value for this factor was relatively close to the 0.05 significance limit. The coefficient for this factor was positive (Figure 4.1), indicating that slightly higher titres were attained at the high setting (1000 µL), thus it was considered worthwhile to investigate whether increasing the fill volume above 1000 µL could produce a real (i.e. statistically significant) improvement in titre. Dox concentration was found to have no impact on titre over the range 1.0 - 5.0 µg mL^{-1} in the second CCF experiment (Section 4.2.1.3), thus a fixed mid-point concentration of 3.0 µg mL^{-1} was used for this experiment. Additionally, a value of 3.0 µg mL^{-1} was chosen rather than 1.0 µg mL^{-1} as some harvests would extend to 46 hr which is beyond the post-induction period examined in the second CCF study. Here then, the influence of post-induction period and liquid fill volume was investigated using a central composite circumscribed (CCC) design, which enabled the impact of each factor to be examined over five levels (Section 4.2.1.1). All other factors were given a fixed value (Table 4.5). The materials and methods employed in this study are detailed in Section 2.4.4. The same responses as had been examined in the previous CCF experiments, plus metabolite concentrations (glucose, lactate,

glutamine and ammonium) and culture pH, were monitored in this study. The design matrix and results are presented in Table 4.6.

Table 4.5 Specification of the factors and settings investigated during the central composite circumscribed (CCC) experiment. Post-induction period and liquid fill volume were investigated over the ranges specified, while the other seven factors were given a fixed value. Ab is an abbreviation for abbreviation.

Factor (units)	Ab	Fixed value	Settings				
			-1.4	-1	0	1	1.4
Shaking speed (rpm)	x_1	160	-	-	-	-	-
Shaking diameter (mm)	x_2	20	-	-	-	-	-
Pre-induction period (hr)	x_3	24	-	-	-	-	-
Post-induction period (hr)	***x_4***	***-***	***22***	***25.5***	***34***	***42.5***	***46***
Liquid fill volume (µL)	***x_5***	***-***	***700***	***788***	***1000***	***1212***	***1300***
Cell seeding density (viable cells mL^{-1})	x_6	1.2×10^6	-	-	-	-	-
Concentration of serum (% [v/v])	x_7	5	-	-	-	-	-
Concentration of dox (µg mL^{-1})	x_8	3.0	-	-	-	-	-
Concentration of NaBu (mM)	x_9	2	-	-	-	-	-

Table 4.6 CCC design matrix for investigation into the effect of post-induction period (x_4) and liquid fill volume (x_5) on cell growth and lentiviral vector production. Each factor was varied over five levels and six centre point experiments were included for estimation of pure error. Factors and their ranges were coded according to Table 4.5. Each run was performed in triplicate and, where relevant, mean values ± 1 SD are presented. Cell concentration and viability measurements were performed immediately prior to lentiviral vector harvests. Predicted values were calculated from the regression model fitted to a given response (see Section 2.4.4), and were computed using the "point prediction" tool within DoE software (similar predicted and measured values are indicative of a well-fitting model).

Run	x_4	x_5	Viable cells mL^{-1} (x 10^6)	Cell viability (%)	RNA copies mL^{-1} (x 10^9)	Titre (x 10^4 TU mL^{-1})		P:I ratio (x 10^5)	
						Measured	Predicted	Measured	Predicted
1	-1	-1	3.5 ± 0.3	95.7 ± 0.6	6.2 ± 0.6	5.4 ± 0.2	6.4	1.1	1.4
2	1	-1	5.1 ± 0.6	92.6 ± 0.5	9.7 ± 2.7	6.3 ± 0.6	7.4	1.5	1.8
3	-1	1	2.1 ± 0.3	93.9 ± 2.0	2.8 ± 1.2	1.2 ± 0.4	1.2	2.3	2.4
4	1	1	3.3 ± 0.4	92.3 ± 0.3	4.2 ± 1.1	1.4 ± 0.8	1.5	3.0	2.8
5	-1.4	0	4.3 ± 0.1	96.7 ± 0.4	5.4 ± 2.1	3.5 ± 0.1	3.2	1.5	1.4
6	1.4	0	5.4 ± 0.1	93.0 ± 0.5	11.0 ± 1.3	5.5 ± 0.3	4.9	2.0	2.0
7	0	-1.4	4.4 ± 0.5	94.2 ± 0.6	11.0 ± 1.8	5.4 ± 0.5	4.7	2.0	1.8
8	0	1.4	2.3 ± 0.2	91.5 ± 0.5	3.0 ± 0.7	1.0 ± 0.4	0.9	3.1	3.2
9	0	0	3.5 ± 0.4	94.2 ± 0.7	6.5 ± 1.0	4.5 ± 0.2	4.5	1.5	1.7
10	0	0	3.0 ± 0.5	94.7 ± 1.0	6.3 ± 1.6	4.5 ± 0.2	4.5	1.4	1.7
11	0	0	3.8 ± 0.1	93.9 ± 0.3	7.5 ± 2.4	3.9 ± 0.5	4.5	1.9	1.7
12	0	0	3.8 ± 0.2	94.2 ± 1.2	9.1 ± 1.1	3.9 ± 0.2	4.5	2.3	1.7
13	0	0	3.4 ± 0.4	93.9 ± 0.3	7.8 ± 1.7	5.0 ± 0.7	4.5	1.6	1.7
14	0	0	3.2 ± 0.2	93.0 ± 0.7	7.9 ± 0.7	5.5 ± 0.8	4.5	1.5	1.7

A quadratic model was fitted to titre data, which had undergone an inverse transformation, and the resulting model yielded an adjR^2 value of 0.99 and predR^2 value of 0.96. The model fit could thus be considered excellent. ANOVA statistics revealed that the model was significant ($p < 0.0001$), and that there was no significant 'lack of fit' ($p = 0.2385$). Post-induction period ($p = 0.0113$), liquid fill volume ($p < 0.0001$) and (liquid fill volume)^2 ($p < 0.0001$) were significant model terms. Note that the terms ([post-induction period] x [liquid fill volume]) and (post-induction period)^2 were also included in the model, although their influence was not significant ($p = 0.1291$ and $p = 0.1672$, respectively). The response surface plot for titre is shown in Figure 4.5. At high liquid fill volumes varying the post-induction period appeared to have no effect on ProSavin® titre, which remained low. It would seem that there is a critical liquid fill volume of approximately 1000 µL, below which higher titres are achieved and post-induction period begins to have an effect on titre. The maximum titre achieved in this experiment was 6.3×10^4 TU mL^{-1} where the post-induction period was 42.5 hr and liquid fill volume was 788 µL (Run 2, Table 4.6). The model was subsequently used to predict optimal values for post-induction period and liquid fill volume, and this work is discussed later (Section 4.2.1.5).

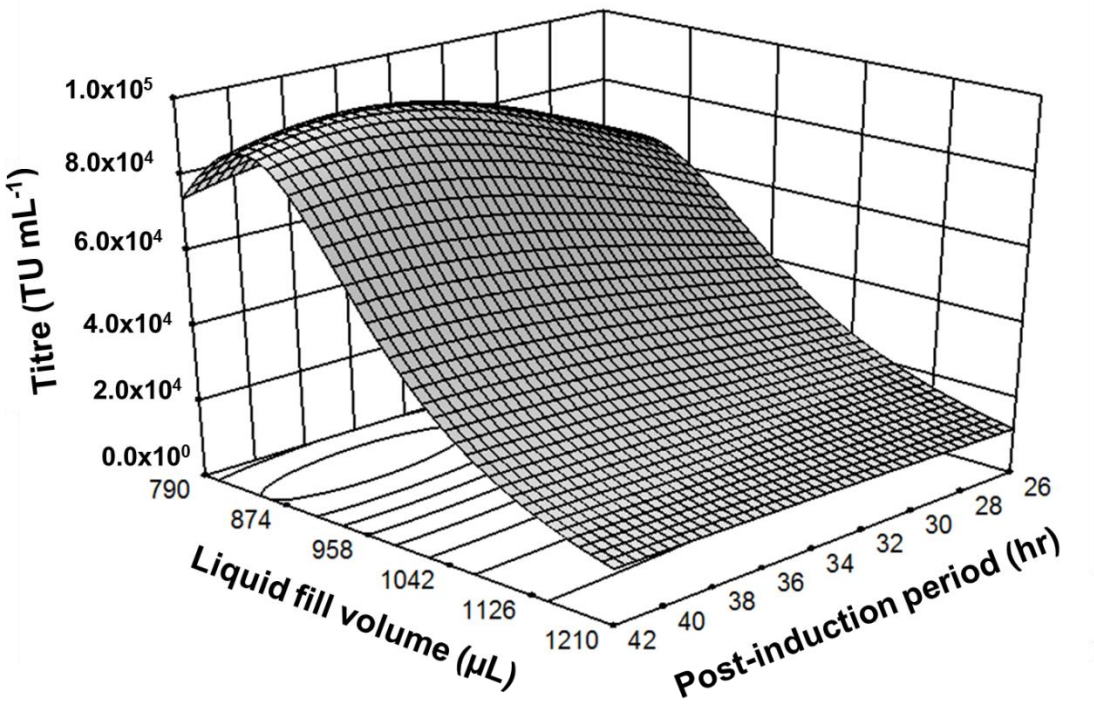


Figure 4.5 Response surface plot displaying the effect of post-induction period and liquid fill volume on titre. This figure was generated using the quadratic model obtained by regression analysis of titre data presented in Table 4.6 (see Equation 4.1). All other operating parameters were fixed as outlined in Table 4.5. This figure was generated using Design Expert software.

The relatively low mean titres attained for experimental runs 3, 4 and 8 (1.0 - 1.4 x 10^4 TU mL^{-1}; Table 4.6) were attributed to poor mixing of the cell culture, due to the high fill volumes utilised (≥ 1212 µL). At these fill volumes, slightly higher lactate concentrations (≥ 2.9 g L^{-1} at the time of harvest) were observed, as compared to experimental runs where the fill volume was ≤ 1000 µL (Figure 4.6 [c]). As expected, the higher lactate concentrations observed for runs 3, 4 and 8 also corresponded to a lower cell culture pH (≤ 6.7 at the time of harvest) as compared to the other experimental runs (Figure 4.6 [a]). Consistent with these findings, it has been previously reported that 2000 µL microwell cultures of Chinese hamster ovary (CHO) cells (generating antibodies) produced more lactate and had a lower pH than 800 µL cultures, when a shaking speed of 120 rpm and shaking diameter of 20 mm was used and the microwell format matched that used in the current work (Barrett *et al.*, 2010). The authors suggested that inadequate oxygen transfer likely triggered this response, as dissolved oxygen levels were reduced to less than 10 % in these cultures (Barrett *et al.*, 2010). It is therefore probable that reduced oxygen

availability, caused by inadequate mixing, instigated the increase in lactate production observed at high fill volumes in the current work. Glucose, lactate and ammonium concentrations were also measured; however no obvious trends were observed in this data (Figure 4.6 [b], [d] and [e]).

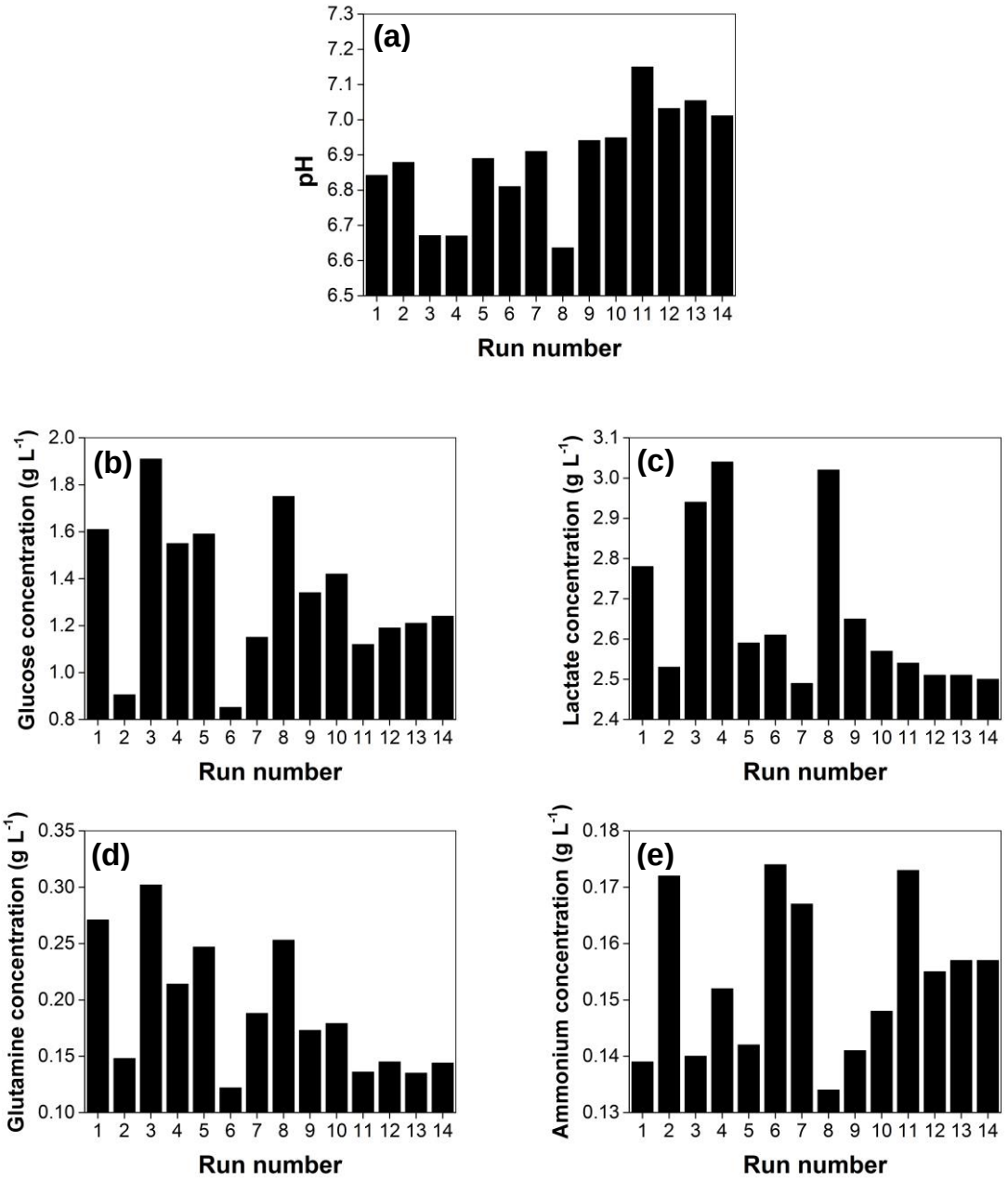


Figure 4.6 The pH (a), glucose concentration (b), lactate concentration (c), glutamine concentration (d), and ammonium concentration (e) of microwell cultures at the time of vector harvests. Details of the operating conditions employed, and the corresponding cell growth, titre and RNA copy number data for each run, is provided in Table 4.6. Although a single measurement was performed to determine pH and metabolite concentrations for each run, a reasonable indication of the natural variability between repeat experiments may be gained from comparing runs 9 to 14 (inclusive), as these were repeated centre point experiments.

A quadratic model was fitted to P:I ratio data, which yielded an adjR^2 value of 0.75 and predR^2 value of 0.62 following model refinement (which was carried out as described in Section 2.4.4). The model fit could therefore be considered good. ANOVA statistics revealed that the model

was significant (p = 0.0007), and that there was no significant 'lack of fit' (p = 0.8519). Liquid fill volume ($p < 0.0006$) and (liquid fill volume)^2 ($p < 0.0046$), but not post-induction period (p = 0.0728), were significant model terms. The P:I ratio response surface plot is shown in Figure 4.7. From this plot it is clear that high fill volumes not only affect titre, but also have a negative effect on the proportion of functional particles in culture supernatants. Post-induction period, on the other hand, did not have a significant impact on the P:I ratio between 26 and 42 hr.

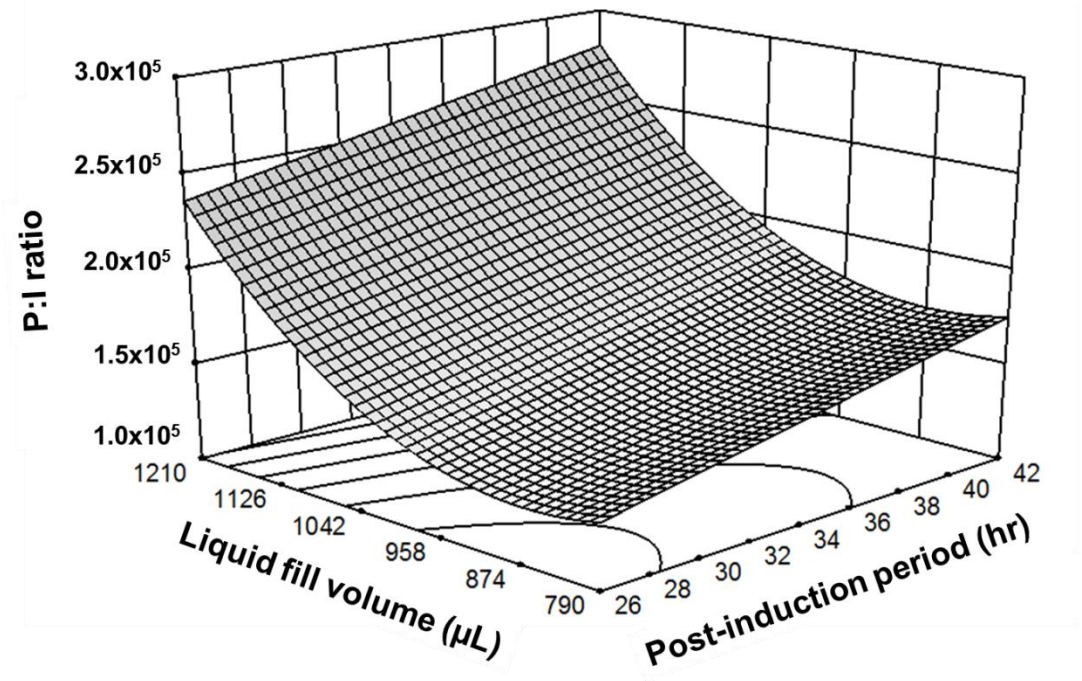


Figure 4.7 Response surface plot displaying the effect of post-induction period and liquid fill volume on the P:I ratio. This figure was generated using a refined quadratic model obtained by regression analysis of the P:I data presented in Table 4.6. All other operating parameters were fixed as outlined in Table 4.5. This figure was generated using Design Expert software.

4.2.1.5 Verification of the predicted optimal values for post-induction period and liquid fill volume

The regression model (vector titre in terms of actual factors) generated from the CCC experimental data (Section 4.2.1.4) took the form:

$$Y^{-1} = 3.38*10^{-4} - (6.54*10^{-7})x_4 - (7.38*10^{-7})x_5 - (1.58*10^{-9})x_4x_5 + (2.61*10^{-8})x_4^{\,2} + (4.68*10^{-10})x_5^{\,2} \quad (4.1)$$

Where: Y = vector titre, x_4 = post-induction period, and x_5 = liquid fill volume. This equation (4.1) was used to predict the optimal factor settings. These were a post-induction period of ≈ 39.7 hr and a liquid fill volume of ≈ 854 µL, for which a titre of 9.3 x 10^4 TU mL^{-1} was predicted. Minimising the P:I ratio was not included as a goal for predicting the optimal factor settings, as the P:I ratio was not affected by post-induction period in the final CCC experiment. In addition, the predicted optimal setting for liquid fill volume based on maximising titre was consistent with previous data and fell within the more favourable lower portion of the response surface plot for P:I ratio (Figure 4.7). A verification experiment was thus performed at these settings (n = 9). All other factors were fixed as for the final CCC experiment (Table 4.5), and the materials and methods for this study are detailed in Section 2.4.5.

The measured P:I ratio was 0.6 x 10^5, lower than had been previously attained up until this point during the DoE experiments (Sections 4.2.1.2 to 4.2.1.4), and the ProSavin® titre was 6.1 ± 0.2 x 10^4 TU mL^{-1}, which was 65 % of the predicted optimum (9.3 x 10^4 TU mL^{-1}). This implied some lack of fit of the model (Figure 4.5). However, in agreement with data obtained during the earlier DoE experiments, harvests performed either side of the supposed optimum harvest time indicated that there is more likely a plateau in titre at around 6 x 10^4 TU mL^{-1}, as opposed to a unique harvest time where a 'peak' in titre can be achieved (Figure 4.8). This 'harvest window' falls approximately between 26 and 46 hr, in agreement with Figure 4.5, as harvests carried out earlier than 26 hr post-induction typically yielded lower titres (Table 4.6 run 5; Figure 4.4; Figure 4.8), as did harvests carried out later than 46 hr post-induction (Table 4.2 runs 2, 4, 6, 8, 10; Figure 4.8). However, this window is unlikely to be fixed, as for example during the first CCF study (Table 4.2), lower titres were observed at 46 hr post-induction than were observed in the final CCC study (Table 4.6), indicating that the precise cut-off for the harvest window may vary slightly between batches. Overall, it may be concluded that the model has accurately predicted the optimum harvest window, but has slightly overestimated the attainable titre.

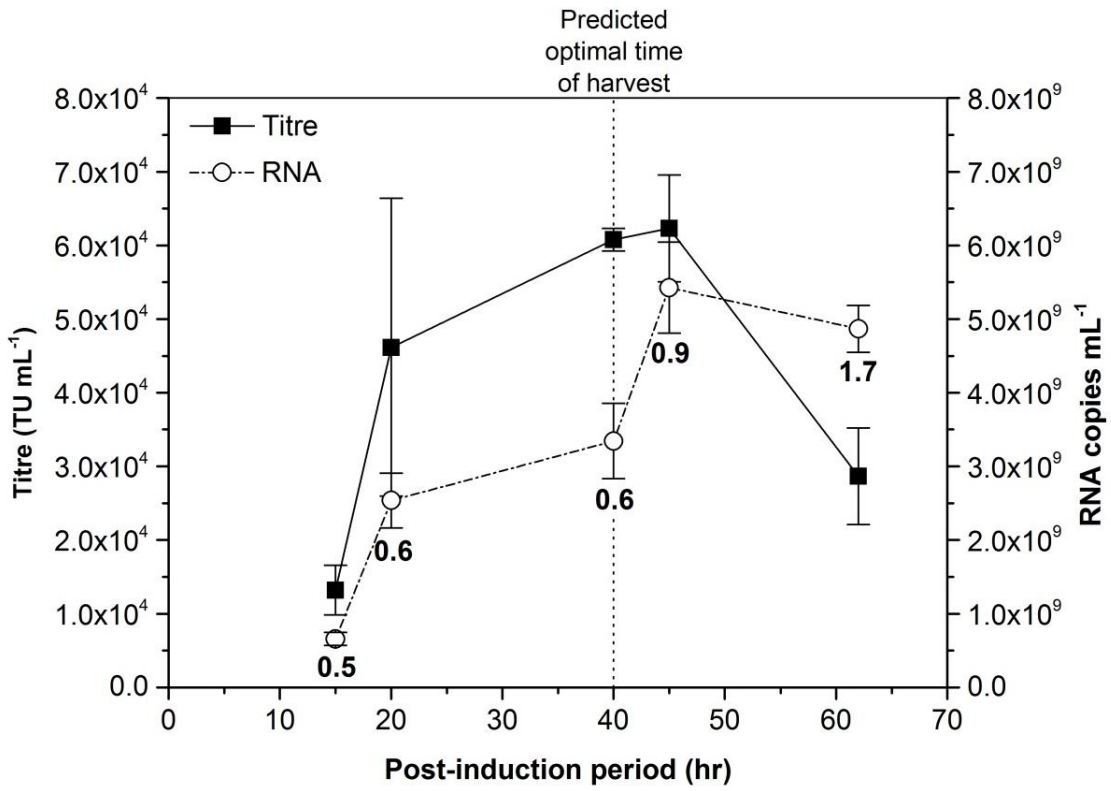


Figure 4.8 ProSavin® titre and RNA copy number in an experiment to evaluate the accuracy of the final central composite circumscribed (CCC) model. Values displayed directly below each pair of data indicate the P:I ratio (x 10^5) for that time point. The liquid fill volume was 854 µL and all other operating parameters were fixed as for the final CCC experiment (Table 4.5). The optimal harvest time (in terms of maximising titre) predicted by the final CCC model (≈ 39.7 hr post-induction) is indicated. Mean values are presented and error bars represent ± 1 SD (n ≥ 3).

Cell growth was also monitored during the verification experiment (Figure 4.9). A drop in viable cell concentration and viability was observed 15 hr post-induction. It is feasible that dox and/or NaBu exerted a cytotoxic impact immediately after their addition, as it was earlier demonstrated that heterogeneities exist temporarily in microwells following small liquid additions (Section 3.2.4), meaning that abnormally high concentrations of these compounds may have briefly come into contact with cells, before being fully dispersed within the bulk fluid. The cells subsequently recovered and a peak in density of 3.6 x 10^6 viable cells mL^{-1} was observed 45 hr post-induction (cell viability remained > 80 % throughout the duration of the study).

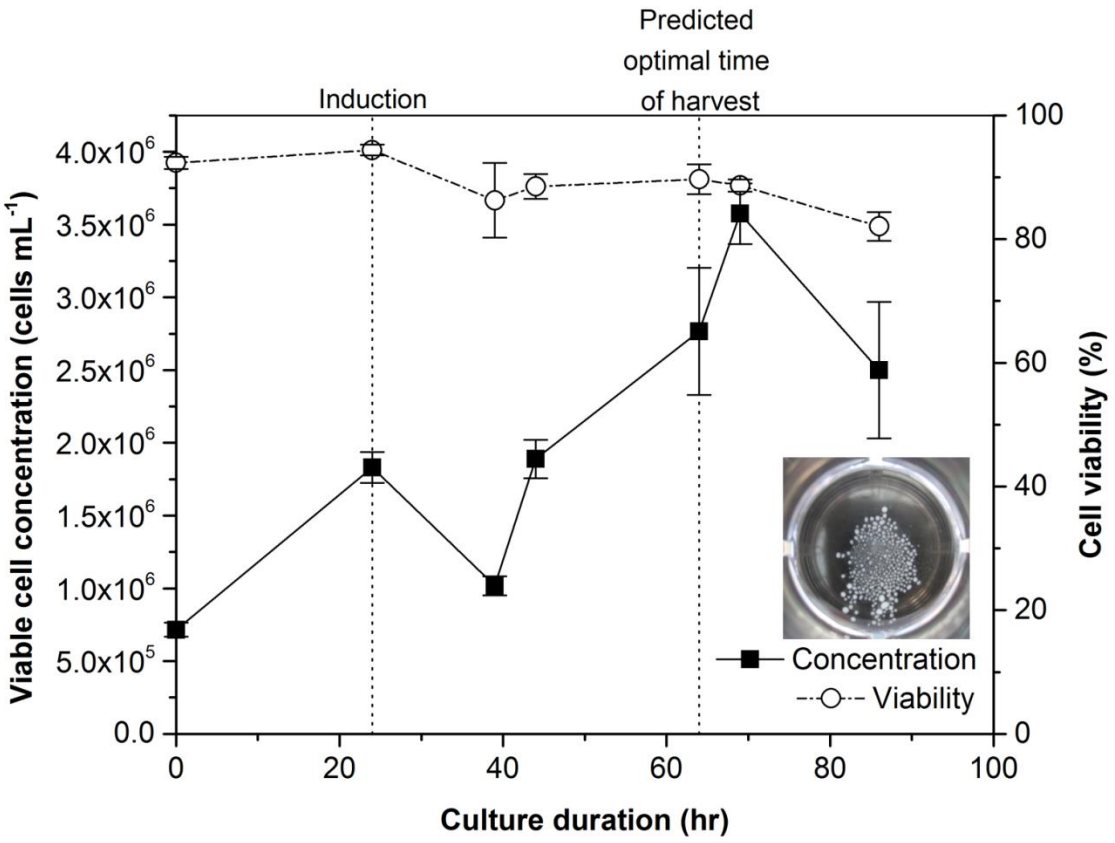


Figure 4.9 The concentration and viability of PS46.2 cells over time, observed during an experiment to evaluate the accuracy of the final CCC model. Operating conditions were as noted for Figure 4.8. The point of cell induction (24 hr) and optimal harvest time (in terms of maximising titre) predicted by the final CCC model (≈ 39.7 hr post-induction) are indicated. Mean values are presented and error bars represent ± 1 SD (n ≥ 3). Insert is a representative photograph of aggregated PS46.2 in a microwell, taken at the end of the culture (90 hr).

Culture pH and osmolality were also monitored throughout the culture period (Figure 4.10). The pH declined from 7.4 at the start of the culture (0 hr) to 6.9 by the supposed optimal harvest time (39.7 hr post-induction / 63.7 hr post-seeding), and remained at 6.9 until the end of the culture (62 hr post-induction / 86 hr post-seeding). This slight acidification of the culture may have negatively impacted on ProSavin® titres, which plateaued and declined after 39.7 hr post-induction (Figure 4.8), as the half-life of lentiviral vectors has been previously reported to decrease rapidly either side of pH 7 (Higashikawa and Chang, 2001). Culture acidification may also have contributed to the reduction in cell concentration and viability observed during the latter stages of the culture (Figure 4.9).

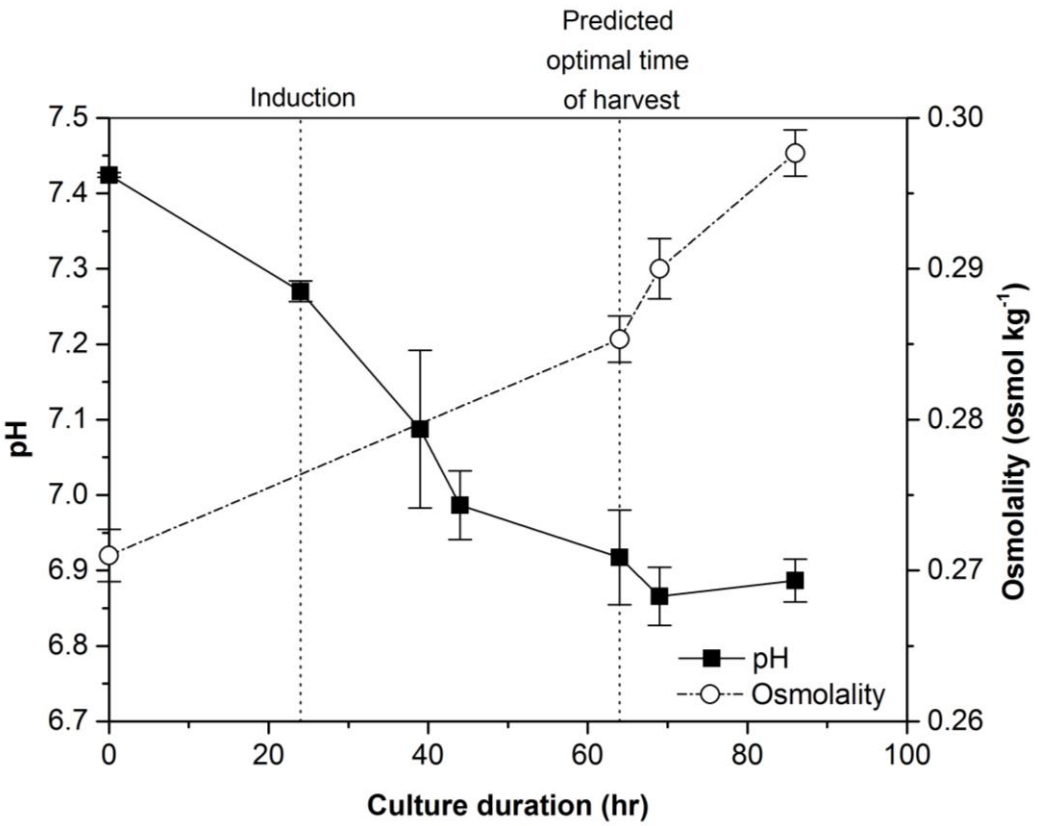


Figure 4.10 The pH and osmolality of PS46.2 cultures over time, observed during an experiment to evaluate the accuracy of the final CCC model. Operating conditions were as noted for Figure 4.8. The point of cell induction (24 hr) and optimal harvest time (in terms of maximising titre) predicted by the final CCC model (≈ 39.7 hr post-induction) are indicated. Mean values are presented and error bars represent ± 1 SD (n = 3).

The osmolality was 0.271 osmol kg^{-1} at the time of seeding (0 hr) and increased slightly throughout the culture, reaching 0.298 osmol kg^{-1} by the end (86 hr post-seeding). This minor increase in osmolality should not have affected PS46.2 cell growth (see earlier discussion in Section 3.2.1.2). To supplement osmolality data, the rate of fluid evaporation from microwells was also determined by gravimetric analysis. Linear regression analysis of this data ($R^2 > 0.99$) revealed that the average rate of fluid loss from microwells was -2.2 % day^{-1}, thus for a starting volume of 854 µL this translates to a loss of -18.8 µL day^{-1}. Based on this data, by the time the culture was terminated (86 hr), the recorded values for cell concentration, titre and RNA copy number were theoretically 8.6 % higher than they would have been, had no evaporation occurred (earlier measurements would have been affected to a lesser degree). Overall, the

extent of evaporation and associated increase in osmolality observed in this study was small, and the impact of these parameters on microwell response data was predicted to be negligible.

4.2.2 Description of liquid phase hydrodynamics in microwells using dimensionless numbers

Momentum, gravitation and surface forces can have a substantial impact on the mixing of fluid in a microwell, which in turn can affect the productivity of lentiviral vector producer cells. The fluid mixing in a microwell can be described using dimensionless numbers (see Section 1.5.3), and by characterising microwells operating under those conditions known to favour high productivity, this information may help to inform subsequent scale-up studies. Using the previously optimised operating conditions for PS46.2 cell culture (i.e. factor settings as described for the verification experiment, Section 4.2.1.5), the Reynolds number (*Re*), Froude number (*Fr*) and Phase number (*Ph*) were calculated as detailed in Section 2.5. The *Re* was calculated to be 932. Microwell *Re* has been previously linked to flow regimes in shaken systems (Barrett *et al.*, 2010), and the relatively low *Re* recorded here indicates that flow was probably laminar. These conditions likely favoured cellular aggregation, such as that observed during the verification experiment (see insert, Figure 4.9). For shaken bioreactors, the *Ph* and *Fr* indicate whether the fluid is following the movement of the shaking platform ("in-phase") or not ("out-of-phase"), and only for conditions where the $Ph > 1.26$ and the $Fr > 0.4$ is "in-phase" operation predicted (Büchs *et al.*, 2000b; Barrett *et al.*, 2010). "In phase" conditions are associated with better mixing and gas transfer. Here, the *Ph* was calculated to be 9.12 and the *Fr* was calculated to be 0.29. Although the *Ph* value was greater than 1.26, the *Fr* value was below the 0.4 value required for efficient mixing. This indicates that the influence of gravity on fluid hydrodynamics cannot be neglected (Lotter and Büchs, 2004). Synonymous with the gravity effects predicted by a low *Fr* was the observation that PS46.2 tended to form aggregates in the central bottom region of the microwells (see insert, Figure 4.9). Overall the data suggests that gentle agitation conditions promote optimal PS46.2 cell growth and ProSavin® production. However, as microwell cultures rely solely on surface aeration for oxygen delivery, these mild mixing conditions may have led to the creation of an oxygen-deprived microenvironment at the base of the microwell when fill volumes greater than approximately 1000 µL were used. Evidence for this may be observed in Table 4.6 and Figure 4.6, where higher lactate

concentrations and a lower pH were associated with high (≥ 1212 µL) fill volume cultures, presumably as a consequence of oxygen limitations (see discussion in Section 4.2.1.4), and this had a detrimental impact on ProSavin® titres and the P:I ratio.

4.2.3 Evaluation of the effect of daily dox additions on ProSavin® titres using microwell cultures of PS46.2

It was demonstrated during the earlier CCF experiments that for harvests performed ≤ 26 hr post-induction, a dox concentration of 1.0 µg mL^{-1} was sufficient for full cell induction, as higher dox concentrations (2.6 - 5.0 µg mL^{-1}) did not yield higher titres (Sections 4.2.1.2 and 4.2.1.3). However, for later harvests (70 hr post-induction), a higher dox concentration (2.6 µg mL^{-1}) did confer a small benefit (Section 4.2.1.2). It was hypothesised that dox may have been lost from the system (e.g. via degradation) over time, meaning that the full induction of cells was not being maintained over extended culture periods when a starting concentration of 1.0 µg mL^{-1} was used. To test this hypothesis, a further microwell experiment was carried out in which the addition of dox at a concentration of 1.0 or 3.0 µg mL^{-1} (added once) was compared to a concentration of 1.0 µg mL^{-1} added every 24 hr. Harvests were performed at 22, 40, 46 and 70 hr post-(first)induction and ProSavin® titres subsequently quantified. The materials and methods for this study are detailed in Section 2.6.

No benefit was observed from supplementing extra dox either as a higher starting concentration of 3.0 µg mL^{-1} or by adding a further 1.0 µg mL^{-1} daily (Figure 4.11). This suggests that the small benefit conferred by a higher dox concentration (2.6 µg mL^{-1} as compared to 1.0 µg mL^{-1}) observed in the first CCF experiment at 70 hr post-induction was likely an artefact of the experimental procedure, caused by the minimal number of repeated experiments employed during the DoE study, and 1.0 µg mL^{-1} is likely sufficient for full cell induction even during this extended timeframe.

It should be noted that, overall, titres were lower in this experiment than were to be expected based on the earlier optimisation experiments (Sections 4.2.1.2 to 4.2.1.5). This was not a result of poorer cell growth as cell densities reached between 3.8 and 4.3 x 10^6 viable cells mL^{-1}, and cell viabilities also remained high (> 82 %) throughout the study (Figure 4.12). For the purpose of monitoring natural process variability, ProSavin® was produced using reference

shake flask cultures, according to the protocol outlined in Section 2.4.1.3, in parallel to all microwell experiments described in this chapter. This data is presented in Table 4.7. For this experiment, reference shake flask titres were two- to three-fold lower than those attained during the earlier optimisation experiments (Table 4.7), indicating that the relatively low microwell titres observed here were likely caused by inherent differences in the cell stock or particular batch of media used. The data collected from reference shake flask cultures demonstrates the typical uncontrolled batch-batch variability associated with the production of ProSavin® using suspension cultures of PS46.2.

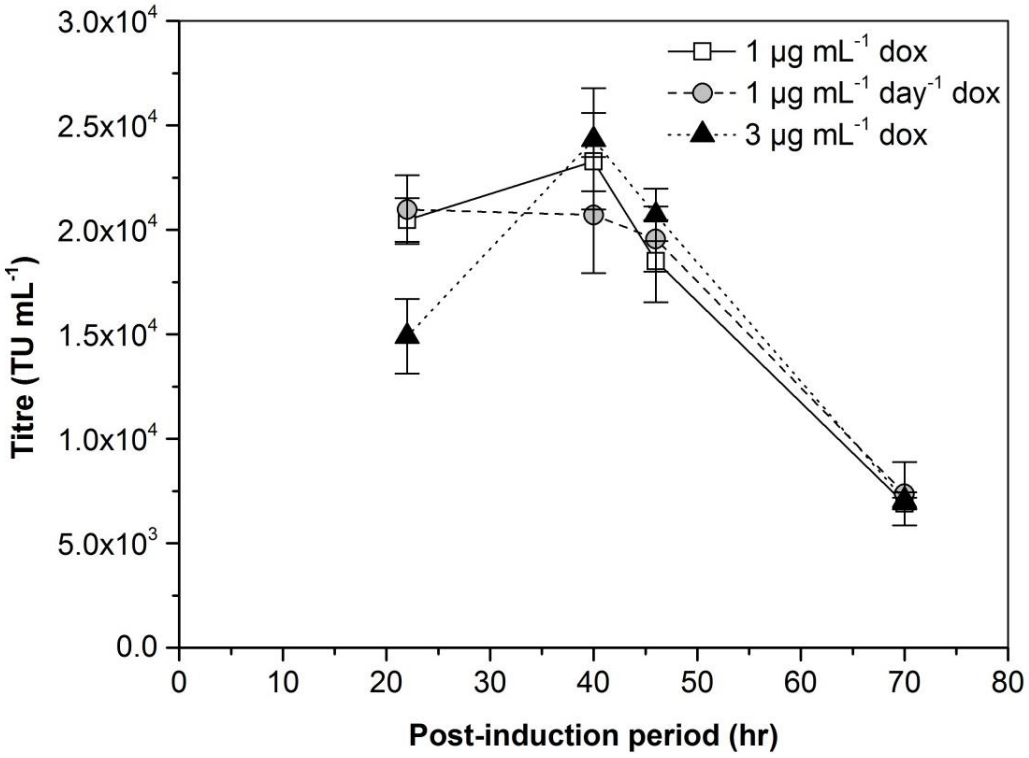


Figure 4.11 The effect of dox supplementation on ProSavin® titres over time. The addition of dox to microwell cultures of PS46.2 at a concentration of 1.0 or 3.0 µg mL^{-1} (added once) was compared to a concentration of 1.0 µg mL^{-1} added every 24 hr. Harvests were performed at 22, 40, 46 and 70 hr post-(first)induction. The liquid fill volume was fixed at 854 µL (as for the verification experiment, Section 4.2.1.5), while the shaking speed, shaking diameter, pre-induction period, cell seeding density, concentration of serum and concentration of NaBu were fixed as for the final CCC experiment (Table 4.5). Mean values are presented and error bars represent ± 1 SD (n = 4).

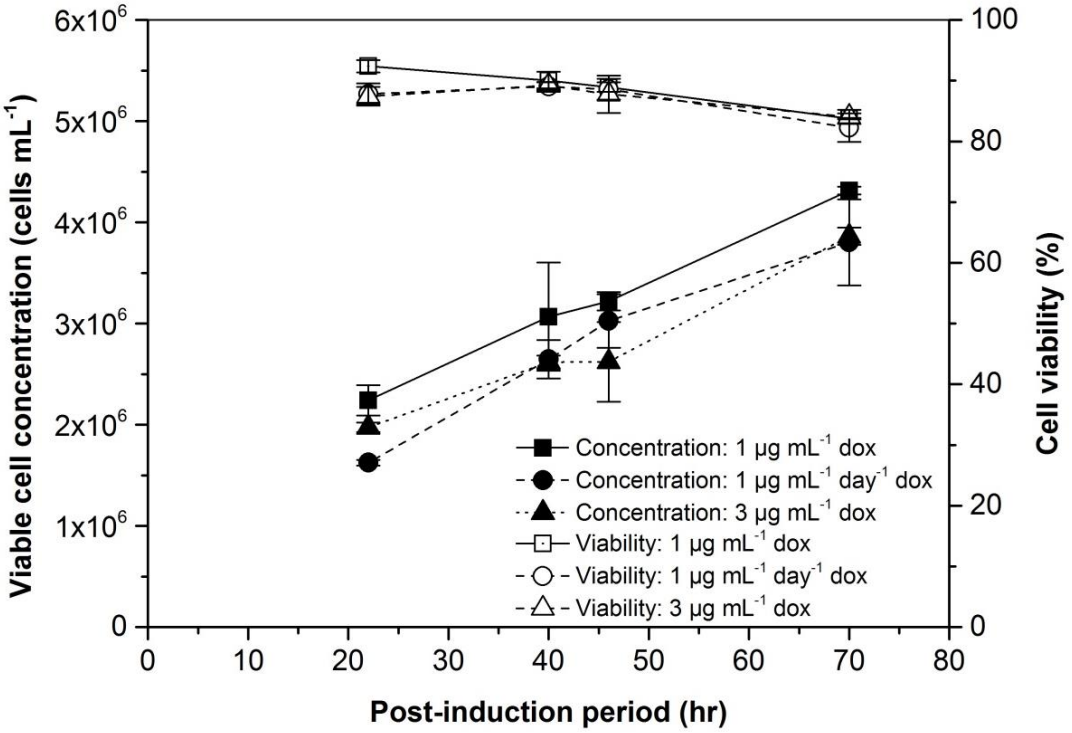


Figure 4.12 The effect of dox supplementation on **t**he concentration and viability of PS46.2 cells over time. The addition of dox to microwell cultures of PS46.2 at a concentration of 1.0 or 3.0 µg mL^{-1} (added once) was compared to a concentration of 1.0 µg mL^{-1} added every 24 hr. Cell concentration and viability were determined immediately prior to vector harvests (which were performed at 22, 40, 46 and 70 hr post-[first]induction). All operating conditions were as stated for Figure 4.11. Mean values are presented and error bars represent ± 1 SD (n = 3).

Table 4.7 Comparison of the maximum microwell titres achieved (for a single set of operating conditions during a single experiment), with titres achieved from reference shake flask cultures run and assayed in parallel. Shake flask culture methods are described in Section 2.4.1.3.

Microwell experiment (section number)	Maximum microwell titre (x 10^4 TU mL^{-1}) (*A*)	Reference shake flask titre (x 10^4 TU mL^{-1}) (*B*)	(*A*) expressed as a % of (*B*)
4.2.1.2	4.0 ± 0.4 (n = 3)	2.0 ± 0.3 (n = 3)	200
4.2.1.3	6.2 ± 1.1 (n = 3)	3.1 ± 0.6 (n = 3)	200
4.2.1.4	6.3 ± 0.6 (n = 3)	2.3 ± 0.4 (n = 3)	273
4.2.1.5	6.2 ± 0.7 (n = 3)	3.2 ± 0.4 (n = 3)	194
4.2.3	2.4 ± 0.2 (n = 4)	1.0 ± 0.1 (n = 3)	240

4.3 Chapter discussion

Using microwell cultures and DoE techniques, an earlier screening experiment (Section 3.2.5) had identified post-induction period, liquid fill volume and concentration of dox as important factors influencing the production of ProSavin® from suspension cultures of PS46.2. Following on from this work, the overall aim of this chapter was to further characterise the influence of these critical factors on ProSavin® production, with a view to optimising production conditions and informing the design of a large scale bioprocess. The first objective was to narrow the operating ranges for post-induction period, liquid fill volume and concentration of dox, and to subsequently predict their optimum settings. This work was described in Sections 4.2.1.1 to 4.2.1.4. The second objective was to verify the predictive capability of the final model. The results of this study were presented in Section 4.2.1.5. The third objective was to describe the fluid mixing within microwells (operating under optimised conditions) using dimensionless numbers. The outcomes of these calculations were described in Section 4.2.2. Finally, the fourth objective was to evaluate whether daily additions of dox to microwell suspension cultures of PS46.2 could improve titres during extended (70 hr) induction periods. This objective stemmed from the earlier results described in Section 4.2.1, and the findings of this study were presented in Section 4.2.3. As the outcome of each individual experiment has already been briefly discussed in the sections mentioned, the purpose here was to bring these discussions together and consider the overall conclusions that may be drawn regarding the impact of harvest timing (Section 4.3.1), microwell mixing (Section 4.3.2), and dox concentration (Section 4.3.3), on the production of ProSavin® from suspension cultures of PS46.2.

4.3.1 The timing of ProSavin® harvests is critical

It was demonstrated that an optimal time frame existed between approximately 26 and 46 hr post-induction, during which maximal titres of around 6 x 10^4 TU mL^{-1} could be attained, representing an approximately two-fold improvement in titre as compared to reference shake flask cultures (Section 4.2.1 and Table 4.7). It is unsurprising that post-induction period was a critical parameter affecting the production of ProSavin® during the batch culture of PS46.2 cells, as vector production is tightly regulated by the tetracycline (tet) repressor (TetR) regulatory protein, which controls expression of both EIAV *gag/pol* and the cytotoxic VSV-G vector

component (Stewart *et al.*, 2009; Stewart *et al.*, 2011). ProSavin® production is thus initiated by the addition of dox, and likely terminated when cell death occurs – potentially as a result of nutrient limitations, such as a lack of glutamine or an accumulation of ammonia (Section 3.2.1.2). It is also possible that cell death may result from VSV-G accumulation. This membrane protein is expressed on the surface of ProSavin® and has been previously reported to have cytotoxic properties (Burns *et al.*, 1993; Yee *et al.*, 1994; Yang *et al.*, 1995; Ory *et al.*, 1996). Titres declined after approximately 46 hr post-induction, when the rate of ProSavin® inactivation exceeded the rate of production. Although a half-life of between 0.8 and 10.4 hr has been reported for HIV-based lentiviral vectors when at 37 °C (Higashikawa and Chang, 2001; Carmo *et al.*, 2009a; Carmo *et al.*, 2009b), the half-life of EIAV-derived lentiviral vectors has not been previously reported, therefore this will be investigated in Chapter 6.

Although a broad region of operability in terms of harvest time was identified (approximately located between 26 and 46 hr), a small amount of variation between experiments was also observed, indicating that the precise cut-off points for this harvest window are likely to be batch specific. Therefore, for the development of a robust manufacturing process, it may be prudent to establish a harvest window which incorporates a margin of safety. Alternatively, further understanding of the relationship between certain process variables and ProSavin® titre may facilitate harvest time to be defined on a batch-by-batch basis. For example, the potential of *in situ* permittivity measurements for real-time detection of lentiviral vector release has recently been demonstrated (Ansorge *et al.*, 2011). The future incorporation of such tools into large scale lentiviral vector manufacturing processes may enable operators to identify a harvest window that is specific for each batch of vector produced, ensuring that yields of lentiviral vectors are of a consistently high titre.

4.3.2 Gentle fluid mixing and low fill volumes promote PS46.2 cell growth and ProSavin® production

The hydrodynamic environment experienced by cells can vary dramatically depending on the type of production vessel and mode of mixing, therefore to inform future scale-up studies, dimensionless numbers were used to describe fluid mixing within microwells. This data revealed that, for microwells operated under optimised conditions, fluid flow was likely laminar

and dominated by gravitational forces, leading to gentle mixing of the culture (Section 4.2.2). Previous data demonstrated that the hydrodynamic forces within microwells can affect the size and arrangement of PS46.2 aggregates (Section 3.2.5.2), and synonymous with the prediction of mild mixing conditions strongly influenced by gravitational forces, was the observation that PS46.2 formed aggregates that collected on the central bottom region of microwells (Figure 4.9 insert). It is unclear whether cellular aggregation limited the productivity of suspension-adapted PS46.2 in this work, as the best titres achieved were approximately seven-fold lower than those reportedly attained when using adherent cultures of PS46.2 (Stewart *et al.*, 2009).

When fill volumes exceeded around 1000 µL, lower pH and higher lactate values were observed, in conjunction with lower ProSavin® titres and a higher P:I ratio (Section 4.2.1.4). As microwell cultures rely solely on surface aeration for oxygen transfer, it is probable that when fill volumes were increased, an oxygen poor microenvironment developed at the base of microwells, and this triggered PS46.2 to increase lactate production. The consequent accumulation of lactate caused acidification of the culture, which in turn likely compromised the productivity of PS46.2 cells, as well as increased the rate of ProSavin® inactivation, as the half-life of lentiviral vectors has previously been reported to decrease rapidly either side of pH 7 (Higashikawa and Chang, 2001). Thus, gentle fluid mixing combined with low (≤ 1000 µL) fill volumes appear to promote ProSavin® production when using microwell suspension cultures of PS46.2.

4.3.3 1 µg mL^{-1} dox is sufficient for the full induction of PS46.2 suspension cells

No data pertaining to the optimal dox concentration required for complete induction of the suspension PS46.2 system was available. PS46.2 incorporates the tet-ON system and, for adherent cultures, 1.0 µg mL^{-1} dox was reported to be sufficient for cell induction (Stewart *et al.*, 2011). As there are obvious differences in the fluid hydrodynamics between shaken *versus* static cell cultures, we investigated whether a higher dox concentration conferred any improvement in ProSavin® titre when using suspension cultures of PS46.2. In-line with reported findings for adherent PS46.2, however, we concluded that a concentration of 1.0 µg mL^{-1} dox was sufficient for full induction of suspension-adapted PS46.2 cells when harvests were performed up to 70 hr post-induction.

4.3.4 Concluding remarks

The use of suspension-adapted stable producer cell lines is highly desired for future large scale productions of clinical grade lentiviral vectors (Ansorge *et al.*, 2010; Schweizer and Merten, 2010; Segura *et al.*, 2013). Here, an approach combining microscale bioprocessing and DoE techniques was used to rapidly characterise the impact of three important factors on the production of ProSavin® (an EIAV-derived lentiviral vector) from suspension cultures of PS46.2 (a stable producer cell line). This approach enhanced our knowledge of the design space, and the data was subsequently used to establish operating conditions for the production of ProSavin® in a WAVE bioreactor as described in Chapter 5. The approach demonstrated here, and the insights gained from its application to ProSavin® bioprocess development, should prove useful to researchers looking to develop a suspension-based process for the manufacture of lentiviral vectors.

5 Scale-up of lentiviral vector production to 2 L WAVE bioreactor scale

5.1 Introduction and aims

To validate the microwell platform (Chapters 3 and 4) for use in future lentiviral vector process development studies, it was necessary to demonstrate that any data generated using the platform was readily scalable. As discussed in Section 1.5.3, no universal strategy exists for scale-up and, for each new system, key process parameters likely to affect product quality require prompt identification and characterisation (Betts and Baganz, 2006; Marques *et al.*, 2010). Scale-up of the ProSavin® process to a wave-mixed bioreactor based on a single criterion, mixing time, was earlier rejected as inappropriate, as values obtained in microwells could not be replicated at large scale (Section 3.2.4) and therefore alternative criteria were sought using a DoE approach. An initial screening experiment in microwells found that ProSavin® titres were significantly affected by post-induction period, liquid fill volume and concentration of dox (Section 3.2.5), and subsequent optimisation studies established approximate operating ranges for these parameters of 26 - 46 hr, 600 - 1000 µL and 1.0 - 5.0 µg mL^{-1}, respectively (Sections 4.2.1 and 4.2.3). Post-induction period was an important parameter as ProSavin® titres are ultimately a function of particle production rates, which vary over time, as well as particle inactivation rates, which curb the accumulation of functional vector in culture supernatants. Liquid fill volume was a critical parameter as it affects microwell mixing and oxygen transfer rates. High fill volumes (≥ 1212 µL) were associated with high lactate concentrations, low pH values and reduced ProSavin® titres (Section 4.2.1.4). Calculation of the dimensionless numbers, *Re*, *Fr*, and *Ph*, revealed that fluid flow in microwells is likely laminar and dominated by gravitational forces, and these mild mixing conditions encouraged the settling of cellular aggregates (Section 4.2.2). As oxygen transfer is achieved via surface aeration, it is likely that when culture volumes exceeded around 1000 µL, an oxygen poor microenvironment developed at the base of microwells, which triggered an increase in cellular lactate production that in turn led to acidification of the culture and compromised titres. Ensuring adequate aeration, while maintaining gentle agitation conditions, was thus crucial for maximising ProSavin® titres when using microwells (note that in a wave-mixed bioreactor, the fill

volume, rocking rate and rocking angle determine the fluid mixing and oxygen transfer characteristics of the culture). Lastly, microwell experiments revealed that a dox concentration of 1.0 µg mL^{-1} was sufficient for full cell induction over typical production timescales (Sections 4.2.1 and 4.2.3), and increasing the concentration to 5.0 µg mL^{-1} had no effect on titres, indicating that this parameter may be given a fixed value within this range. The overall aim of this chapter was to discover if the insights obtained at the microwell scale were relevant upon scale-up of the process to a wave-mixed bioreactor, in order to verify whether (or not) the microwell platform could be usefully employed in the future to support the development of lentiviral vector processes.

As discussed earlier (Section 1.4.2), the development of scalable lentiviral vector manufacturing processes is an area urgently requiring attention (Ansorge *et al.*, 2010; Schweizer and Merten, 2010; Segura *et al.*, 2013). To date, just four production strategies based on suspension cell culture have been developed and trialled using laboratory scale (2.3 - 3.0 L working volume) stirred tank (Segura *et al.*, 2007; Broussau *et al.*, 2008; Ansorge *et al.*, 2009) or disposable wave-mixed (Witting *et al.*, 2012) bioreactors. Although one of these approaches used a tet-ON inducible packaging cell line (Broussau *et al.*, 2008), the remaining three approaches relied solely on the use of transient transfection techniques (Segura *et al.*, 2007; Ansorge *et al.*, 2009; Witting *et al.*, 2012) making them ill-suited to the generation of licensed product (see summary Table 1.9 and Sections 1.4.1 and 1.4.2 for more information). In this chapter, a fifth process, based on the suspension culture of tet-ON stable producer cells using a laboratory scale WAVE bioreactor (GE Healthcare) was developed. A WAVE bioreactor, rather than a conventional stirred tank bioreactor, was chosen for evaluation, as this system provides culture conditions that are most representative of those found to be optimal within microwells (see Section 4.2.2), that is, a low shear environment characterised by bubble-free aeration and laminar flow (Oncül *et al.*, 2010; Eibl *et al.*, 2010b). The specific objectives of this chapter are:

- To use the insights gained at the microwell scale (Chapters 3 and 4) to establish a basic operating protocol for ProSavin® production in a 2 L WAVE bioreactor.
- To evaluate the impact of rocking rate and post-induction period on culture performance in order to verify whether the key conclusions generated using the microwell system (i.e. oxygenation plus gentle agitation are critical; optimal time to harvest is

approximately 26 - 46 hr post-induction) hold relevance following scale-up of the process to a WAVE bioreactor.

Results included in Section 5.2.1 of this chapter were published in: Guy, H. M., *et al*. (2013). *Human Gene Therapy Methods*, **24(2)**: 125-139. Permission to reproduce this content has been granted by Mary Ann Liebert, Inc.

5.2 Results

5.2.1 Evaluation of a 2 L WAVE bioreactor system for ProSavin® production

5.2.1.1 Initial feasibility study using standard operating conditions

The first objective of this work was to establish a working protocol for ProSavin® production in a 20/50 WAVE bioreactor (GE Healthcare), fitted with a 2 L disposable Cellbag (GE Healthcare). A full description of the system employed is provided in Section 2.7.1. An initial pilot run was performed using the system in which the choice of key operating parameters: cell seeding density, time to induction and concentration of inducer compounds, was based on optimised microwell conditions, while harvest timings reflected those evaluated during the final microwell verification experiment (Section 4.2.1.5). The initial liquid fill volume chosen was the maximum 1 L working volume (50 %) recommended by the manufacturer, as this enabled the full capacity of the system to be exploited. Further decisions regarding bioreactor-specific variables such as rocking rate, rocking angle, air flow rate and starting CO_2 concentration were made using the manufacturer's recommendations in conjunction with available literature pertaining to HEK293(T) cell growth and/or virus production in wave-mixed bioreactors (Singh, 1999; Genzel *et al.*, 2006; Wernli *et al.*, 2008; Throm *et al.*, 2009; Greene *et al.*, 2012; Witting *et al.*, 2012). Based on earlier observations of gentle fluid mixing in the microwell system (Section 4.2.2), a relatively slow rocking rate of 10 rocks min^{-1} was chosen corresponding to the lowest rocking rate reported for the cultivation of suspension cells in reviewed literature. Suspension-adapted PS46.2 cells were used to seed the WAVE bioreactor and, for comparison, parallel microwell plates (which were operated under optimised conditions). Cells were induced after 24 hr, and ProSavin® harvests were performed at 17, 23, 40, 47 and 65 hr post-induction (± 1 hr).

Samples were collected throughout the culture period for analysis of cell growth, culture pH and ProSavin® titre. A full description of the protocol employed is provided in Section 2.7.2.

Cell growth and viability data is presented in Figure 5.1. Although both the WAVE bioreactor and microwell cultures were seeded using the same cell stock (prepared at a density of 1.2 x 10^6 viable cells mL^{-1}), immediately post-seeding the viable cell concentration within the WAVE bioreactor was 75 % (9.4 x 10^5 viable cells mL^{-1}) of that recorded for parallel microwell cultures (1.3 x 10^6 viable cells mL^{-1}). Likely as a direct consequence of this lower starting cell density, the viable cell concentration within the WAVE bioreactor subsequently remained consistently between 61 and 76 % of that recorded for parallel microwell cultures (Figure 5.1). Cell viability data obtained from the WAVE bioreactor and parallel microwells was similar, with viabilities remaining above 80 % throughout the culture (Figure 5.1). Cellular aggregation was observed in the WAVE bioreactor, however, the size and extent of aggregates was generally similar to that observed in parallel microwells (Figure 4.9 insert).

The slightly lower cell recoveries observed for the WAVE bioreactor culture immediately post-seeding were probably caused by the method of inoculation used. The cells were syringed into the Cellbag through a length of C-Flex tubing® that extended into a tissue culture hood, as at this point no sterile tubing welder was available for use. While it is unlikely that cell losses arose as a consequence of shear damage inflicted by this approach (cell viabilities were > 90 % and comparable to those observed in microwells at 0 hr), it is possible that some cells were left in the tubing. A different approach for seeding the Cellbag was adopted for the later studies described in Sections 5.2.1.2 and 5.2.2, which involved flushing the inoculum addition line with media after the addition of cells (details of the method are provided in Sections 2.7.3.2 and 2.7.3.3). This appeared to resolve the issue for subsequent WAVE runs (Figures 5.3 and 5.6), as starting cell concentrations were 1.0 - 1.2 x 10^6 viable cells mL^{-1} (86 - 100 % of the target density) in all instances except where the Cellbag had been pre-coated with media (starting cell concentration was 6.9 x 10^5 viable cells mL^{-1}; 58 % of the target density), which could have been due to rapid adherence of cells to the vessel interior in this experiment (Section 5.2.1.2).

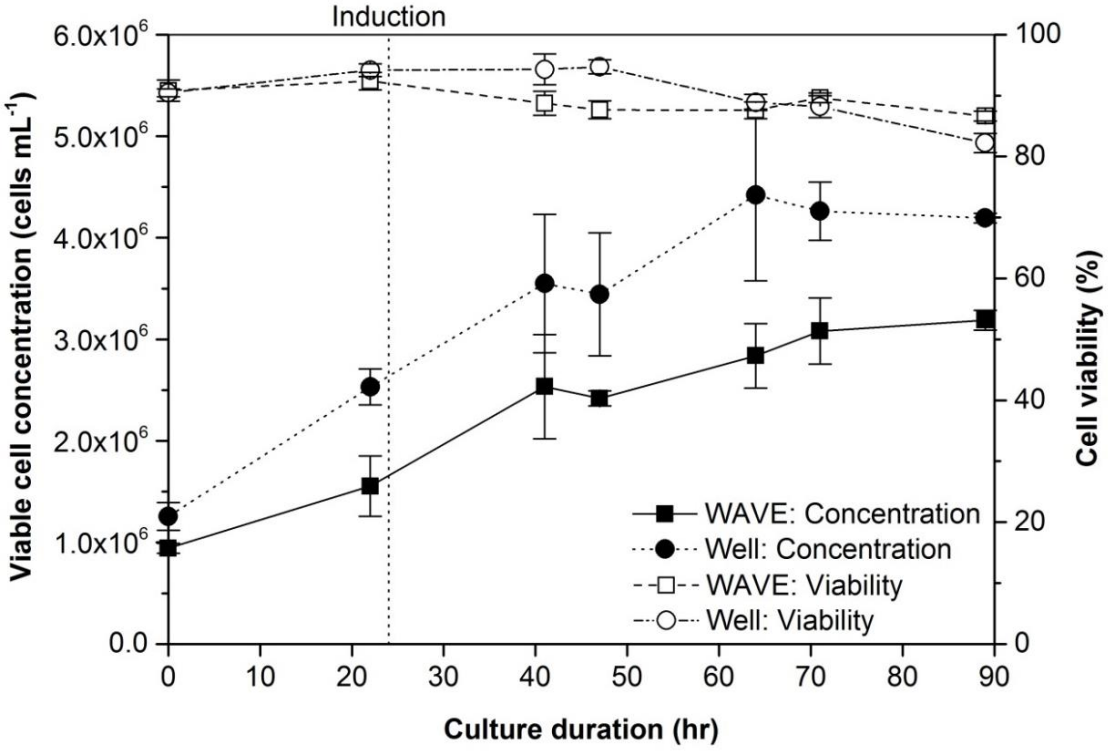


Figure 5.1 Growth and viability of PS46.2 cells cultured in a WAVE bioreactor using a Cellbag with 50 % (1 L) fill volume (n = 1). Data from parallel reference microwell cultures of PS46.2 is also shown (n = 3). Production of ProSavin® was induced after 24 hr, as indicated on the graph. The materials and methods for this study are described in Section 2.7.2. Mean data is plotted and error bars represent ± 1 SD (sample material obtained from the WAVE culture was subjected to three repeat measurements, from which the mean and SD were calculated, while microwell data represents three measurements taken from three separate wells).

Despite the reasonable growth and high viability of PS46.2 when cultured within the WAVE bioreactor (Figure 5.1), ProSavin® titres were relatively low (Figure 5.2). The best titre achieved in the WAVE bioreactor was just 8.9 x 10^3 TU mL^{-1} (40 hr post-induction), which was 12 % of the best titre achieved in parallel microwells (7.2 x 10^4 TU mL^{-1}; 23 hr post-induction). It was noted during the earlier microwell experiments that when fill volumes of greater than 1000 µL were used, cellular lactate production was elevated (presumably due to oxygen limitations as oxygen transfer is facilitated by surface aeration only within microwells), which led to acidification of the medium and a reduction in ProSavin® titres (see Section 4.2.1.4). Like microwell cultures, WAVE bioreactor cultures rely on surface aeration for oxygen transfer, and thus may also be subjected to oxygen restrictions under certain operating conditions. In this study, the pH within the WAVE bioreactor dropped from 7.0 to 6.7 after 41 hr, and remained at

6.7 until termination of the culture, while the pH within microwells stabilised at 7.0 (Table 5.1). Earlier microwell studies had demonstrated that relatively small decreases in the culture pH can be symptomatic of large reductions in ProSavin® titres: cultures with a fill volume above 1 mL yielded pH values of 6.7 and titres four- to six-fold lower than equivalent cultures with a fill volume less than or equal to 1 mL and pH of between 6.8 and 7.2 (Section 4.2.1.4). The low pH within the WAVE bioreactor was indicative of oxygen limited conditions caused by inadequate mixing of the culture. The combined use of a low rocking rate (10 rocks min^{-1}) and high fill volume (1 L) was likely responsible for this effect.

Finally, culture osmolality (Section 2.11.1.2) was measured at one time point (40 hr post-induction) to verify that evaporation was not an issue when using the WAVE bioreactor. The recorded value was 0.277 osmol kg^{-1} (n = 1), while for parallel microwells the osmolality was 0.278 osmol kg^{-1} (n = 1; combined material from triplicate wells). Both of these values are well within the normal range expected during suspension culture of PS46.2 (see Section 3.2.1.2), and consequently this parameter was not investigated further.

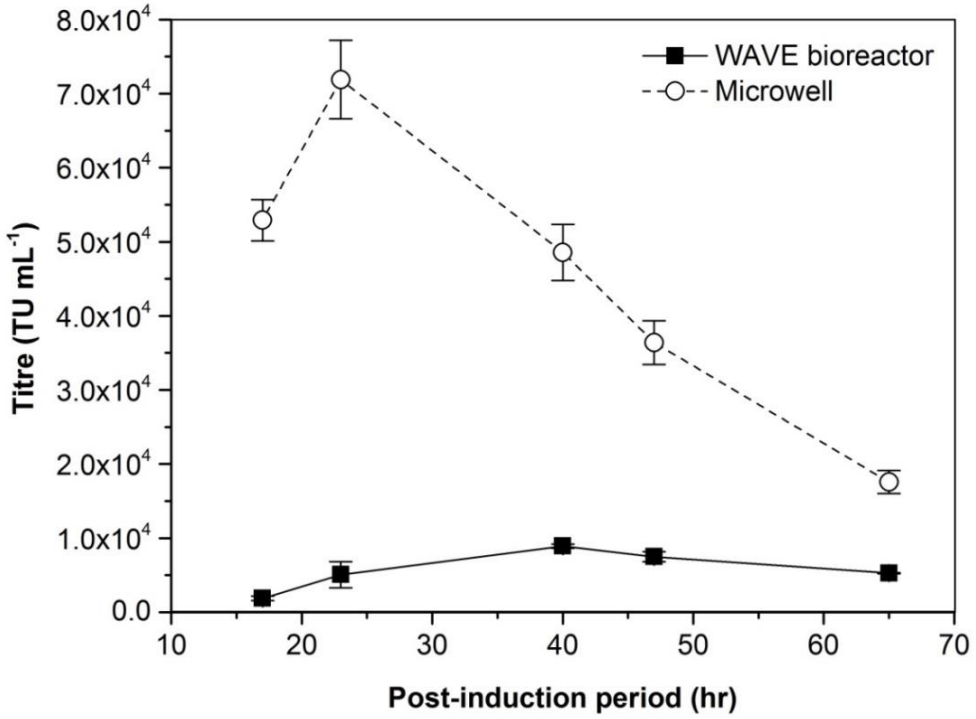


Figure 5.2 ProSavin® titres (measured using the rapid ProSavin® titre assay; Section 2.11.2.1) obtained from PS46.2 cells cultured in a WAVE bioreactor using a Cellbag with 50 % (1 L) fill volume (n = 1). Data from parallel reference microwell cultures of PS46.2 is also shown (n = 3). The materials and methods for this study are described in Section 2.7.2. Mean data is plotted and error bars represent ± 1 SD (sample material obtained from the WAVE culture was subjected to three repeat measurements, from which the mean and SD were calculated, while microwell data represents three measurements taken from three separate wells).

Table 5.1 pH measured at various time points during the culture of PS46.2 cells in a WAVE bioreactor using a Cellbag with 50 % (1 L) fill volume (n = 1). Data from parallel reference microwell cultures of PS46.2 is also shown (n = 1). Production of ProSavin® was induced after 24 hr. The materials and methods for this study are described in Section 2.7.2. pH measurements were performed at the time points noted ± 1 hr.

Culture duration (hr)	Post-induction period (hr)	pH	
		WAVE bioreactor	Microwell
0	-	7.7	7.7
22	-	7.3	7.3
41	17	6.7	7.0
47	23	6.7	7.0
64	40	6.7	7.0
71	47	6.7	7.0
89	65	6.7	7.0

5.2.1.2 Refinement of the operating procedure based on results obtained at the microwell scale

The first experiment using the WAVE bioreactor yielded a relatively low maximum titre (Section 5.2.1.1), thus some modifications to the procedure were made and subsequently trialled in a second evaluative study. Based on the discovery during microwell experiments that maintaining a relatively low liquid fill volume was critical for maximising ProSavin® yields (Section 4.2.1.4), the working volume of the Cellbag was reduced to 0.5 L, which is 25 % of the total volume (equivalent to 850 µL in the microwell system). A further modification was that the CO_2 concentration was reduced to 1 % (from 5 %) after 24 hr culture (at the time of induction), to help retain the culture pH at approximately pH 7. Finally, the impact of pre-coating the Cellbag was explored. The manufacturer recommends pre-coating the Cellbag when chemically-defined lipid supplements are used, as the internal surface of the culture chamber can sequester these compounds from the culture medium (General Electric Company, 2008b). Although such supplements are not used during suspension culture of PS46.2, it was unknown whether any of the components of FreeStyle™ 293 Expression Medium or tet-free FCS were susceptible to depletion in this manner. It was thus deemed prudent to investigate whether pre-coating the bag proffered any benefit for cell growth or vector production.

In this experiment, two Cellbags were therefore operated in parallel where one Cellbag was pre-coated and the other was not. Suspension-adapted PS46.2 cells were used to seed both Cellbags and, for comparison, parallel microwell plates, which were operated under optimised conditions. Cells were induced after 24 hr, and ProSavin® harvests were performed at 18, 22, 41, 47 and 66 hr post-induction (± 1 hr). Samples were collected throughout the culture period for analysis of cell growth, culture pH and ProSavin® titre. Oxygen saturation was also monitored in the Cellbag that had been pre-coated, and RNA copy number measurements were performed using supernatants from the Cellbag that had not been pre-coated. A full description of the protocol employed is provided in Section 2.7.3.2.

Cell growth and viability data is presented in Figure 5.3. Pre-coating the Cellbag had a detrimental effect on both cell growth and viability. In the pre-coated Cellbag, the maximum cell concentration recorded was 2.6×10^6 viable cells mL^{-1}, which was reached after 90 hr. By this point the cell viability had dropped to just 60.6 %. In the Cellbag that had not been pre-coated,

the cell concentration peaked earlier (after 71 hr culture) and was 48 % higher (3.9×10^6 viable cells mL^{-1}). The cell viability was also consistently higher throughout the culture (Figure 5.3), and after 90 hr was 69.6 %. It was surprising that pre-coating the Cellbag had such a marked negative impact on PS46.2 cell growth and viability. During the study, obvious differences in the aggregative behaviour of PS46.2 were noted between the two Cellbags. In the Cellbag that had not been pre-coated, cellular aggregation was similar to that observed in the first experiment (Section 5.2.1.1). In the pre-coated Cellbag, however, the extent of aggregation was severe, and large masses of cells adhered to the base of the Cellbag at the periphery of the rocked liquid flow, where the cells were routinely exposed to the air-liquid interface. It may be reasonably conjectured that this phenomena was responsible for the relatively low cell numbers and reduced viability of this culture. It is possible that, in pre-coating the Cellbag, some components from the tet-free FCS or media allowed the cells to form a biofilm and aggregate. The cell concentration and viability of parallel microwell cultures were higher than in either of the WAVE bioreactor cultures (Figure 5.3). It should be noted, however, that the maximum cell concentration recorded here (6.3×10^6 viable cells mL^{-1} at 65 hr) was exceptional, and that in previous microwell experiments (Chapter 4) the cell concentrations recorded were typically closer to those recorded in the Cellbag that was not pre-coated (e.g. compare Figure 4.9 and Figure 5.3).

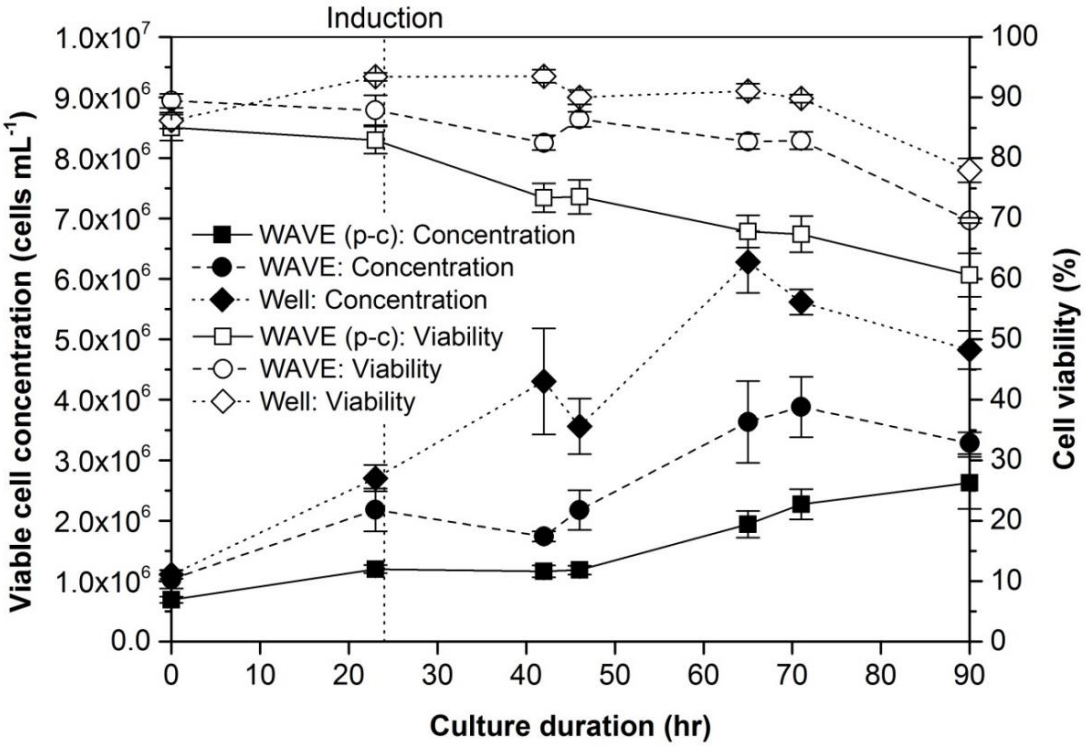


Figure 5.3 Growth and viability of PS46.2 cells cultured in a WAVE bioreactor using two Cellbags, each with a 25 % (0.5 L) fill volume. For comparison, one Cellbag was pre-coated (p-c) with media (n = 1), while the other was not (n = 1). Data from parallel reference microwell cultures of PS46.2 is also shown (n = 3). Production of ProSavin® was induced after 24 hr, as indicated on the graph. The materials and methods for this study are described in Section 2.7.3.2. Mean data is plotted and error bars represent ± 1 SD (sample material obtained from each WAVE culture was subjected to three repeat measurements, from which the mean and SD were calculated, while microwell data represents three measurements taken from three separate wells).

ProSavin® titre data displayed a similar trend to cell data. WAVE bioreactor titres attained from the Cellbag that was not pre-coated consistently exceeded those achieved from the Cellbag that was pre-coated (Figure 5.4). The relatively low titres recorded for the pre-coated Cellbag were likely a direct result of the poorer cell growth and viability within this culture due to cellular aggregation effects. Encouragingly, however, the maximum titres achieved from both WAVE bioreactor cultures were higher than those observed during the previous study (Section 5.2.1.1). The maximum titre achieved from the Cellbag that was not pre-coated was 2.5 x 10^4 TU mL^{-1} (41 hr post-induction), which was 47 % of the maximum titre achieved in parallel microwells (5.2 x 10^4 TU mL^{-1}; 22 hr post-induction). RNA copy numbers were also quantified using

supernatants from this Cellbag, and this data was used in conjunction with titre data to calculate particle:infectivity (P:I) ratio values for each time point (P:I ratio = RNA copy number / titre). This data is also included in Figure 5.4. As was observed during the earlier microwell optimisation studies (Section 4.2.1), culture supernatants harvested from the WAVE bioreactor contained an increasing proportion of defective particles over time and by 41 hr post-induction, when the maximum titre was recorded, the P:I ratio was 1.1 x 10^5 (Figure 5.4). This is in reasonable agreement with the P:I ratio of 0.9 x 10^5 recorded in the earlier microwell verification experiment at 45 hr post-induction (when the maximum titre was recorded; Figure 4.8).

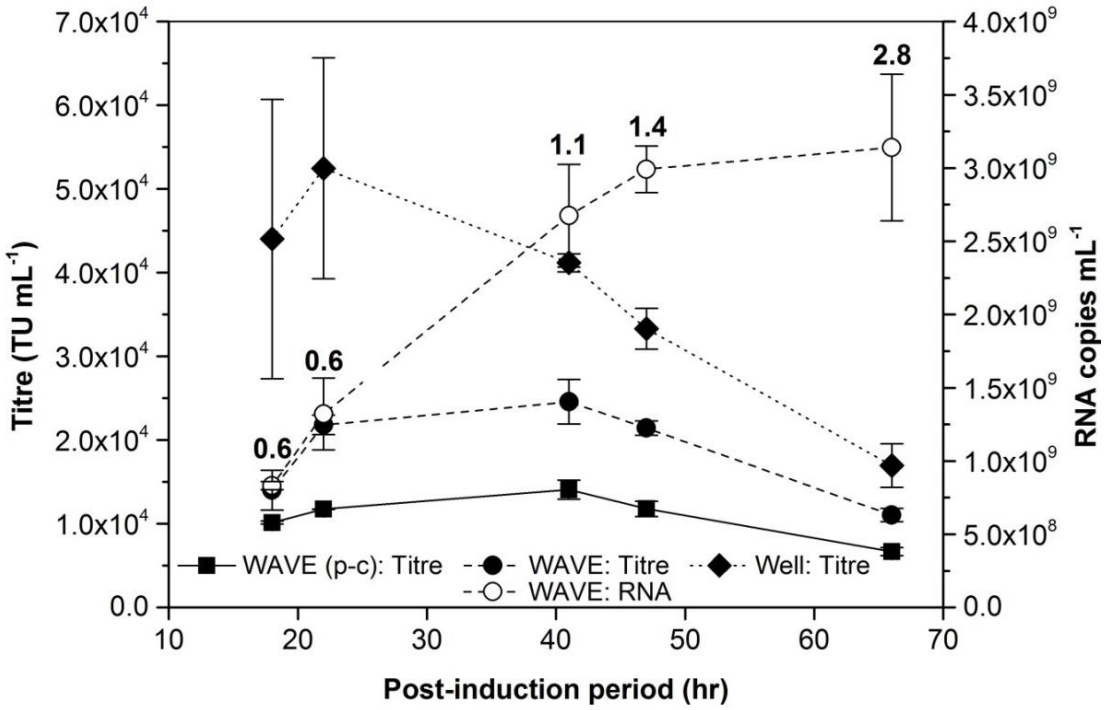


Figure 5.4 ProSavin® titres obtained from PS46.2 cells cultured in a WAVE bioreactor using two Cellbags, each with a 25 % (0.5 L) fill volume. For comparison, one Cellbag was pre-coated (p-c) with media (n = 1), while the other was not (n = 1). Data from parallel reference microwell cultures of PS46.2 is also shown (n = 3). RNA copy number values are also presented for one WAVE culture (Cellbag *not* pre-coated), and the particle:infectivity (P:I) ratio (x 10^5) is also displayed directly above each RNA data point. The materials and methods for this study are described in Section 2.7.3.2. Mean data is plotted and error bars represent ± 1 SD (sample material obtained from each WAVE culture was subjected to three repeat measurements, from which the mean and SD were calculated, while microwell data represents three measurements taken from three separate wells).

In this study, two key modifications to the initial WAVE bioreactor operating protocol (Section 5.2.1.1) were made: (i) the working volume of the Cellbag was reduced, and (ii) the concentration of CO_2 pumped through the headspace was lowered during the latter stages of the culture. It is clear that using a 25 % Cellbag fill volume is more conducive to ProSavin® production than using a 50 % fill volume via comparison of Figures 5.4 and 5.2. Here, the pH in both Cellbags, as well as in microwells, stabilised at 7.0 (Table 5.2). This suggests that the cultures were adequately mixed and did not experience oxygen limited conditions, such as likely occurred when a 50 % fill volume was used. Consistent with this hypothesis was the observation that *in situ* oxygen saturation levels in the pre-coated Cellbag remained reasonably high (> 57 %) throughout the culture period (Table 5.3). However, it should be noted that, as well as the reduction in fill volume, the concentration of CO_2 mixed with air and pumped through the headspace was also reduced (from 5 to 1 % after 24 hr) in this experiment, and it is possible this modification to the procedure could also have contributed to stabilise the pH and improve titres as CO_2 blending can have a acidifying effect on cell culture media. Overall, it seems unlikely that the 5 % CO_2 concentration employed for the duration of the 50 % fill volume WAVE bioreactor study (Section 5.2.1.1) caused the observed acidification of the culture after 41 hr. In this earlier study (Section 5.2.1.1), parallel microwell cultures were also exposed to a 5 % CO_2 atmosphere, yet a similar drop in culture pH was not observed. In addition, the Freestyle™ 293 expression medium used for this work is buffered for use in an 8 % CO_2 atmosphere. This suggests that lactate build-up (caused by a limited availability of oxygen) instigated the reduction in pH observed in the earlier experiment (Section 5.2.1.1) and lowering the fill volume counteracted this effect in the current study. Overall, a working strategy has been developed for ProSavin® production using a WAVE bioreactor, which yields titres within two to three-fold and a P:I ratio comparable to those commonly achieved in microwells.

Table 5.2 pH measured at various time points during the culture of PS46.2 cells in a WAVE bioreactor using two Cellbags, each with a 25 % (0.5 L) fill volume. For comparison, one Cellbag was pre-coated (p-c) with media (n = 1), while the other was not (n = 1). Data from parallel reference microwell cultures of PS46.2 is also shown (n = 1). Production of ProSavin® was induced after 24 hr. The materials and methods for this study are described in Section 2.7.3.2. pH measurements were performed at the time points noted ± 1 hr.

Culture duration (hr)	Post-induction period (hr)	pH		
		WAVE bioreactor (p-c)	WAVE bioreactor	Microwell
0	-	7.7	7.7	7.7
23	-	7.3	7.0	7.3
42	18	7.0	7.0	7.0
46	22	7.0	7.0	7.0
65	41	7.0	7.0	7.0
71	47	7.0	7.0	7.0
90	66	7.0	7.0	7.0

Table 5.3 Oxygen saturation recorded at various time points during the culture of PS46.2 cells in a WAVE bioreactor using a Cellbag with 25 % (0.5 L) fill volume. The Cellbag had been pre-coated with media (note: before being replaced with inoculum, this media served to calibrate the oxygen probe). Production of ProSavin® was induced after 24 hr. The materials and methods for this study are described in Section 2.7.3.2. Oxygen saturation values were recorded at the time points noted ± 1 hr.

Culture duration (hr)	Post-induction period (hr)	Oxygen saturation (%)
0	-	94.8
16	-	77.7
23	-	74.9
42	18	57.7
46	22	58.8
65	41	63.9
71	47	70.2
90	66	79.8

5.2.2 Investigation into the effect of WAVE bioreactor rocking rate and post-induction period on ProSavin® production to establish the accuracy of microwell predictions

Following proof-of-principle studies (Section 5.2.1), a reasonable operating procedure had been established for ProSavin® production using the WAVE bioreactor, although titres remained lower than those achieved using the microwell system. Earlier microwell experiments had demonstrated that manipulation of the culture mixing conditions was crucial for maximising ProSavin® yields; high fill volumes had led to reduced titres (see Sections 3.2.5.1 and 4.2.1.4).

Thus, this study examined whether altering the culture mixing conditions could also improve titres generated using the WAVE system. Rocking rate, rather than fill volume, was varied in order to produce different mixing conditions within the WAVE bioreactor, as it was undesirable to further reduce the (already low) working volume. Eight separate bioreactor runs were performed, using rocking rates of 6, 12, 18 and 24 rocks min^{-1} (n = 2). According to the manufacturer a rocking rate of 6 rocks min^{-1} is generally the minimum required for bulk mixing and particle suspension in a WAVE bioreactor (General Electric Company, 2008a). Rocking rates of between 6 and 15 rocks min^{-1} have been employed for the culture of anchorage-dependent HEK293-derived cell lines (General Electric Company, 2008a; Throm *et al.*, 2009; Greene *et al.*, 2012), while rocking rates of 10 to 22 rocks min^{-1} have been used for the culture of suspension-adapted HEK293-derivatives (Singh, 1999; Wernli *et al.*, 2008). To maximise the likelihood of detecting conditions optimal for PS46.2 growth and ProSavin® production, a broad range of rocking rates were consequently evaluated in the current study that encompassed those previously employed for the successful maintenance of HEK293-based cells (Singh, 1999; Wernli *et al.*, 2008; General Electric Company, 2008a; Throm *et al.*, 2009; Greene *et al.*, 2012). Earlier microwell experiments had also demonstrated the critical impact of harvest timing on ProSavin® titres, therefore multiple harvests were performed during each run.

In brief, the WAVE bioreactor was seeded with suspension-adapted PS46.2 cells, which were induced after 24 hr, and ProSavin® harvests were performed at 17, 23, 41, 47 and 65 hr post-induction. Samples were collected throughout the culture period for the analysis of various parameters (see Sections 5.2.2.1 to 5.2.2.5 below). To monitor process variability, ProSavin® was produced using reference shake flask cultures parallel to all WAVE runs, and this data is presented in Section 5.2.2.6. A full description of the protocol employed is provided in Section 2.7.3.3.

5.2.2.1 Cell growth kinetics

Viable cell concentration data is displayed in Figures 5.5 and 5.6. Figure 5.5 illustrates the extent of variability between duplicate WAVE bioreactor runs. Overall, cell growth was similar between duplicate runs, although the data collected during the first run performed at 12 rocks min^{-1} (Figure 5.5 [b], [1]) and the second run performed at 18 rocks min^{-1} (Figure 5.5 [c], [2])

appears somewhat erratic. This was attributed to cellular aggregation, which, except for when a rocking rate of 6 rocks min^{-1} was used, was reasonably extensive during all runs. Typically, some cells adhered to the Cellbag, while those in solution formed a combination of large, loosely aggregated (i.e 'stringy') clumps, and smaller spherical clumps. The rocking rate was increased immediately prior to sample removal during all WAVE runs to aid representative sampling (see Section 2.7.2.2). While this did help to create a more homogenous cell population, large clumps of cells often remained intact and, their inclusion (or not) in cell count samples may have skewed measurement data. When a rocking rate of 6 rocks min^{-1} was used, cellular aggregates were much finer, giving the appearance of suspended silt, and no cells adhered to the Cellbag, thus a representative sample for cell counts was more easily obtained. It is unclear why cellular aggregation was more extensive when higher rocking rates (12 - 24 rocks min^{-1}) were used, although it was established during the earlier microwell studies (Section 3.2.5.2) that the hydrodynamic environment can impact on PS46.2 aggregate formation. A modified Reynolds number (Re_{mod}) has been previously developed and used to describe fluid flow in a 2 L WAVE bioreactor (Eibl and Eibl, 2006; Eibl *et al.*, 2010b). However, insufficient information was reported to enable calculation of this parameter for our system as, for example, the equation incorporated a correction factor (C) for which values were not provided (Eibl and Eibl, 2006). Despite this, a reasonable indication of the fluid conditions likely prevailing during the culture of PS46.2 cells could be obtained from scrutinising the Re_{mod} data presented by Eibl and Eibl (2006). For a 2 L Cellbag operating with a 10 - 50 % liquid fill volume, it was reported that turbulent flow predominated when the Re_{mod} exceeded around 600 (Eibl and Eibl, 2006; Eibl *et al.*, 2010b). When the liquid fill volume and rocking angle were fixed at 50 % and 6°, respectively, the Re_{mod} was close to 600 when a rocking rate of 6 rocks min^{-1} was used, but was significantly higher (> 1000) when a rocking rate of 12, 18 or 24 rocks min^{-1} was employed (Eibl and Eibl, 2006). It is likely that the Re_{mod} values for our system differed slightly to these due to the use of a lower 25 % fill volume. Despite this, the data indicate that runs conducted using a rocking rate of 12, 18 or 24 rocks min^{-1} were most likely characterised by turbulent conditions, while cultures undertaken using a rocking rate of 6 rocks min^{-1} may have experienced laminar flow. It is possible that cell-to-cell and cell-to-Cellbag collisions occurred at a relatively higher frequency and velocity when the rocking rate equalled or exceeded 12 rocks min^{-1}, thus opportunities for aggregate formation were increased. To assess the impact of rocking rate on

cell growth, viable cell concentration data from duplicate runs was combined (Figure 5.6). Rocking rate had no obvious impact on cell growth, with cells reaching a similar maximum density (3.0 - 5.2 x 10^6 viable cells mL^{-1}) at a similar stage of the culture (65 - 89 hr post-seeding) in all runs (n = 8).

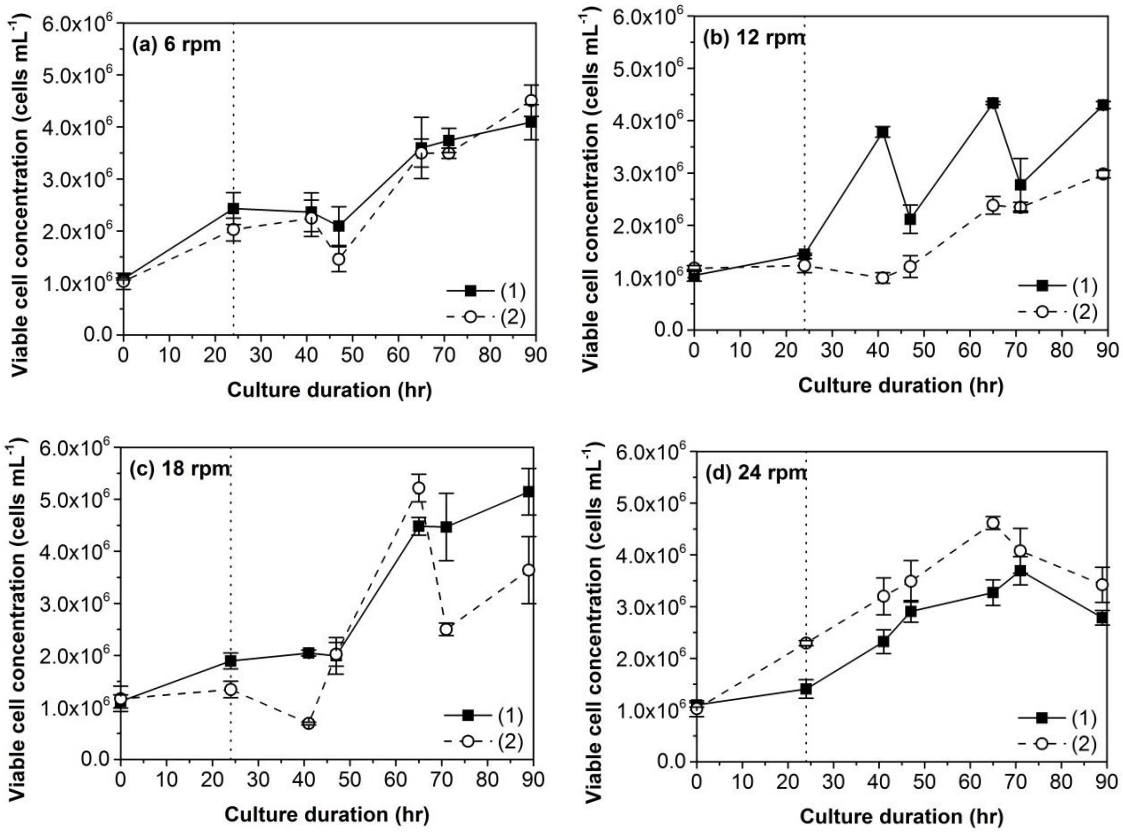


Figure 5.5 Growth of PS46.2 cells in a WAVE bioreactor rocked at a rate of (a) 6 rpm, (b) 12 rpm, (c) 18 rpm, or (d) 24 rpm, where 'rpm' is an abbreviation for rocks min^{-1}. Cells were cultured using a Cellbag with 25 % (0.5 L) fill volume, and production of ProSavin® was induced after 24 hr, as indicated by the dotted vertical line. Materials and methods for this study are described in full in Section 2.7.3.3. Eight separate bioreactor runs were performed (two at each rocking condition), and data from individual runs is presented (at each time point, sample material was subjected to three repeat measurements, and the mean ± 1 SD is displayed).

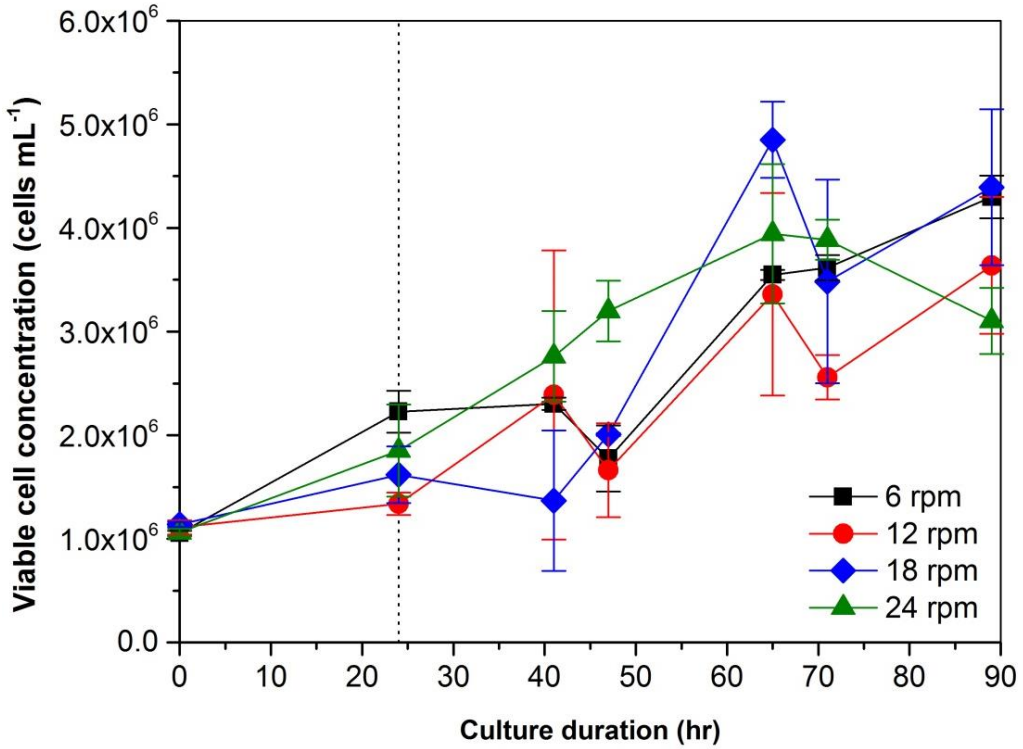


Figure 5.6 Summary graph illustrating the growth of PS46.2 cells in a WAVE bioreactor rocked at a rate of 6, 12, 18, or 24 rpm (where 'rpm' is an abbreviation for rocks min^{-1}). Production of ProSavin® was induced after 24 hr, as indicated by the dotted vertical line. Operating conditions were as noted for Figure 5.5. Eight separate bioreactor runs were performed (two at each rocking condition), and mean data from duplicate runs is presented (error bars represent the range).

Cell viability data is presented in Figures 5.7 and 5.8. Data from duplicate runs was comparable (Figure 5.7), indicating that there is little variability between repeated WAVE bioreactor runs with regards to cell survival. Note that, as viability measurements are expressed as a proportion (%), they are not liable to be skewed by the over- or under-representation of cellular aggregates within a sample. Cell survival was affected by rocking rate (Figure 5.8). When a rocking rate of 6 rocks min^{-1} was used, cell viabilities remained high, measuring between 86.1 and 86.3 % at the termination of the culture (89 hr post-seeding). When a rocking rate of 12, 18 or 24 rocks min^{-1} was used, however, viabilities were relatively lower by the end of the culture period, measuring between 58.8 and 73.8 %. It has previously been reported that power input typically increases with rocking rate over the range 6 to 24 rocks min^{-1} (Eibl *et al.*, 2010b). Consequently, cells cultured using a rocking rate of 6 rock min^{-1} were likely subjected to the

lowest level of hydrodynamic stress in the current study, and this may account for their relatively higher rate of survival.

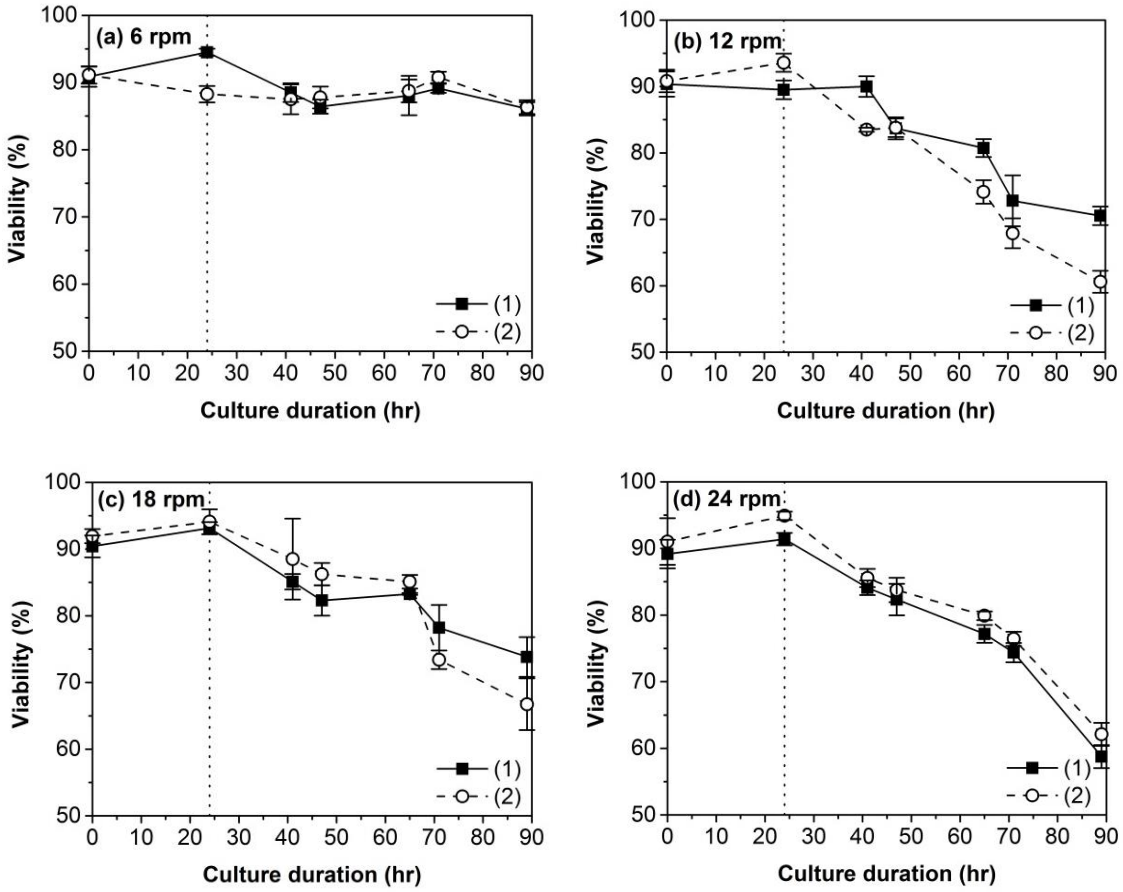


Figure 5.7 Viability of PS46.2 cells in a WAVE bioreactor rocked at a rate of (a) 6 rpm, (b) 12 rpm, (c) 18 rpm, or (d) 24 rpm, where 'rpm' is an abbreviation for rocks min^{-1}. Production of ProSavin® was induced after 24 hr, as indicated by the dotted vertical line. Operating conditions were as noted for Figure 5.5. Eight separate bioreactor runs were performed (two at each rocking condition), and data from individual runs is presented (at each time point, sample material was subjected to three repeat measurements, and the mean ± 1 SD is displayed).

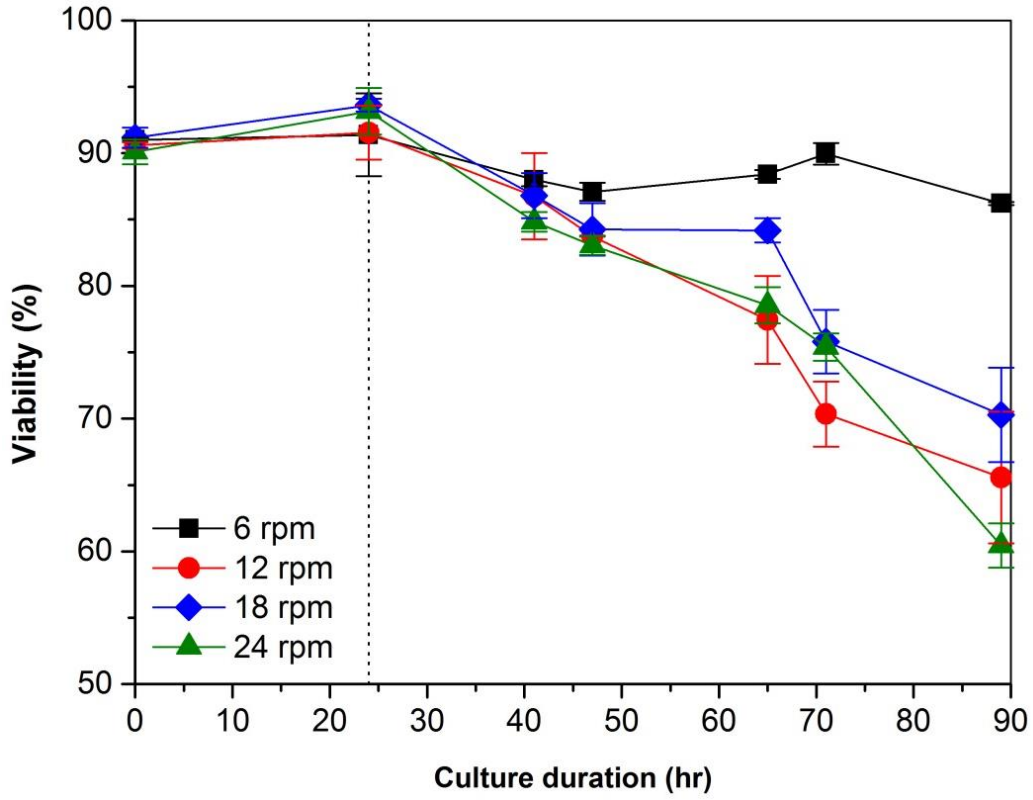


Figure 5.8 Summary graph illustrating the viability of PS46.2 cells in a WAVE bioreactor rocked at a rate of 6, 12, 18, or 24 rpm (where 'rpm' is an abbreviation for rocks min^{-1}). Production of ProSavin® was induced after 24 hr, as indicated by the dotted vertical line. Operating conditions were as noted for Figure 5.5. Eight separate bioreactor runs were performed (two at each rocking condition), and mean data from duplicate runs is presented (error bars represent the range).

5.2.2.2 Dissolved oxygen and pH data

In situ dissolved oxygen was monitored during the bioreactor runs, as described in Section 2.11.1.5. Data collected during duplicate runs was comparable (Figure 5.9), indicating that there is little variability between WAVE bioreactor runs performed at the same rocking rate in terms of cellular oxygen demands. Oxygen saturation levels remained highest (≥ 81.1 %) during the runs performed at 24 rocks min^{-1}, and declined in line with decreasing rocking rates (Figure 5.10), with levels falling as low as 36.8 and 27.7 % during the runs performed at 6 rocks min^{-1}. As viable cell concentrations, and therefore likely cellular oxygen requirements, were similar during all runs regardless of rocking rate (Figure 5.6), this indicates that the oxygen transfer rate (k_La) increased in line with rocking rate. This is consistent with previous reports that k_La is a function of rocking rate (Singh, 1999; General Electric Company, 2008a). Finally, it

has previously been reported that, during the continuous culture of hybridoma cells, cell viabilities increased as dissolved oxygen levels decreased, presumably because oxidative damage to cellular components was reduced (Miller *et al.*, 1987). It is worth considering, therefore, whether the low oxygen levels observed during the WAVE runs performed at 6 rocks min^{-1} (Figure 5.10) could also have contributed to the higher cell viabilities of these cultures as compared to the other runs (Figure 5.8).

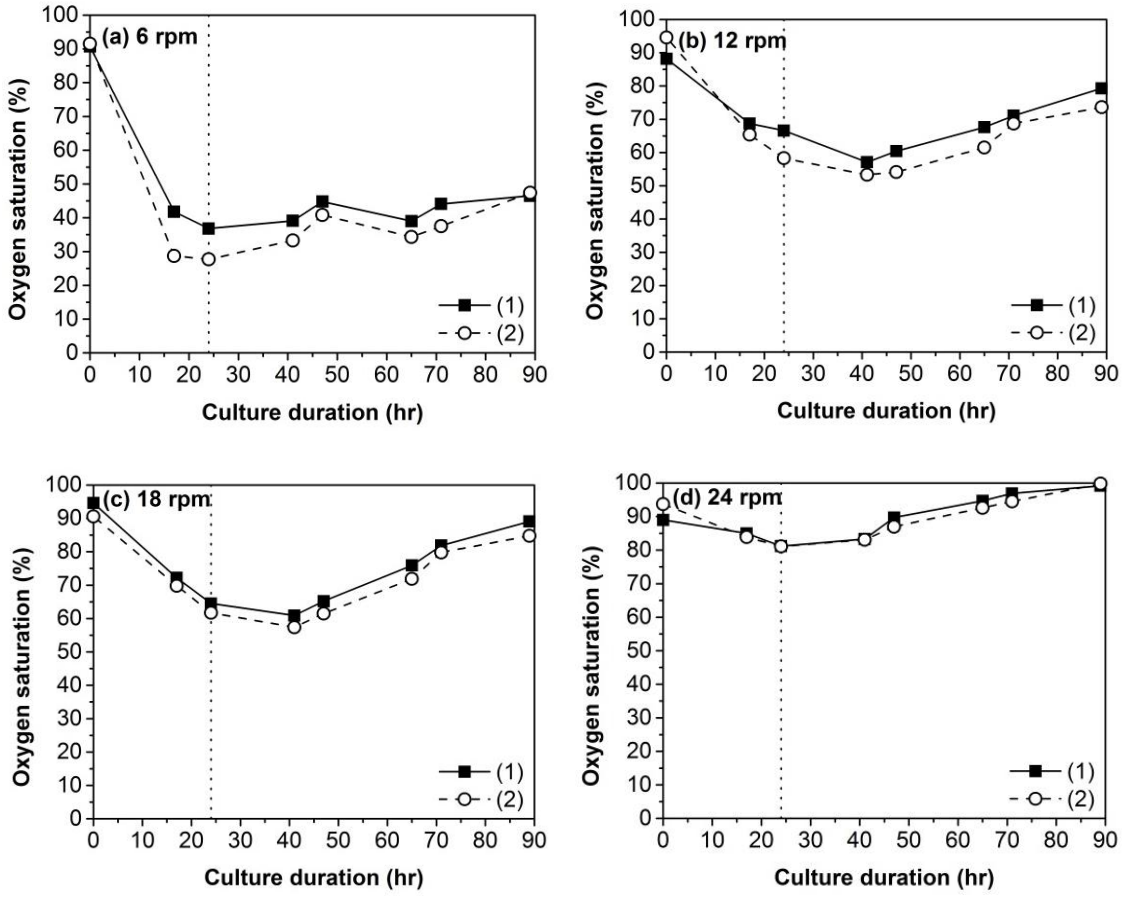


Figure 5.9 Oxygen saturation of PS46.2 cultures in a WAVE bioreactor rocked at a rate of (a) 6 rpm, (b) 12 rpm, (c) 18 rpm, or (d) 24 rpm, where 'rpm' is an abbreviation for rocks min^{-1}. Production of ProSavin® was induced after 24 hr, as indicated by the dotted vertical line. Operating conditions were as noted for Figure 5.5. Eight separate bioreactor runs were performed (two at each rocking condition), and data from individual runs is presented (n = 1).

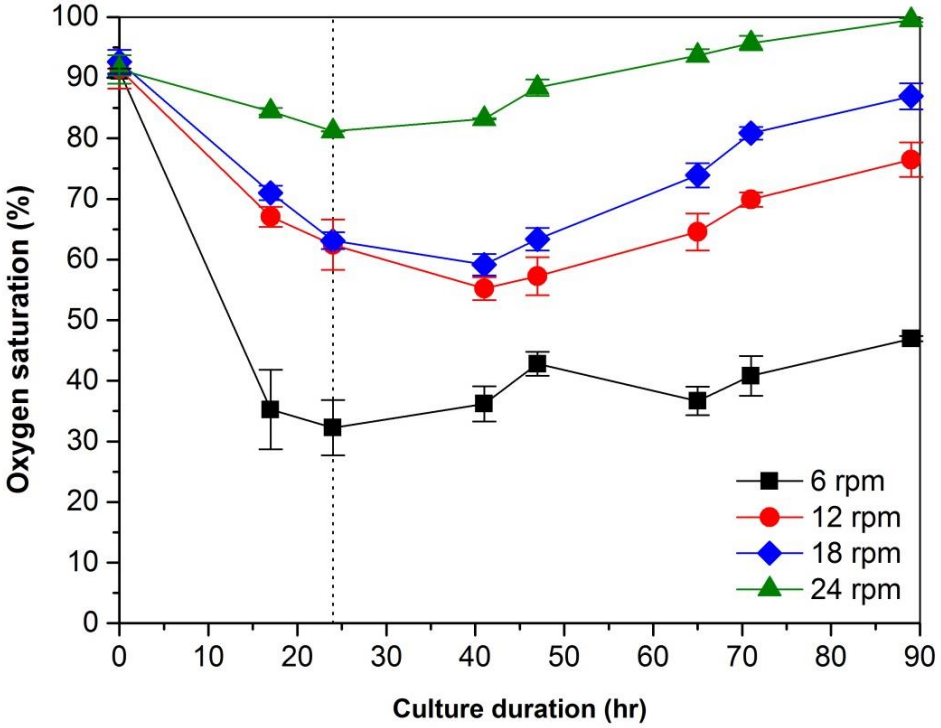


Figure 5.10 Summary graph illustrating the oxygen saturation of PS46.2 cells in a WAVE bioreactor rocked at a rate of 6, 12, 18, or 24 rpm (where 'rpm' is an abbreviation for rocks min^{-1}). Production of ProSavin® was induced after 24 hr, as indicated by the dotted vertical line. Operating conditions were as noted for Figure 5.5. Eight separate bioreactor runs were performed (two at each rocking condition), and data from duplicate runs is presented (error bars represent the range).

Culture pH was monitored during all WAVE bioreactor runs using pH indicator strips as described in Section 2.11.1.3. This data is presented in Table 5.4. Data from the runs conducted using rocking rates of 12, 18 or 24 rocks min^{-1} were similar, with the pH typically stabilising at 7.0. Only pH data collected during the runs performed at 6 rocks min^{-1} was notably different. Consistent with the earlier microwell (Section 4.2.1.4) and WAVE bioreactor (Section 5.2.1.1) findings that poor mixing leads to acidification of the culture, the pH fell as low as 6.4 (47 hr post-seeding) during the runs performed at 6 rocks min^{-1}. This effect was likely produced by increased lactic acid production as a result of limited oxygen availability (see earlier discussions in Sections 4.2.1.4 and 5.2.1.1), and consistent with this supposition was the observation that dissolved oxygen levels were also lowest for these cultures (Figure 5.10). As the optimum dissolved oxygen level for mammalian cell growth has been reported to be around 50 % of saturation with air (Miller *et al.*, 1987), it is reasonable to suppose that the runs

performed using a rocking rate of 6 rocks min^{-1} were oxygen limited (Figure 5.10). Although the observed reduction in dissolved oxygen did not negatively impact on the growth or viability of PS46.2 cultures (Section 5.2.2.1), the associated fall in culture pH (Table 5.4) indicates that the cells responded to these lower oxygen levels by increasing lactic acid production. The culture pH subsequently recovered slightly and stabilised at 6.7 (Table 5.4), indicating that the cells may have switched to lactate consumption during the latter stages of the culture, as was previously observed (see Sections 3.2.1.2 and 3.2.1.3).

Table 5.4 pH measured at various time points during the culture of PS46.2 cells in a WAVE bioreactor rocked at a rate of 6, 12, 18, or 24 rpm (where 'rpm' is an abbreviation for rocks min^{-1}). Production of ProSavin® was induced after 24 hr. Operating conditions were as noted for Figure 5.5. Eight separate bioreactor runs were performed (two at each rocking condition), and data from individual runs is presented (n = 1).

Culture duration (hr)	Post-induction period (hr)	pH							
		6 rpm		12 rpm		18 rpm		24 rpm	
		(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
0	-	7.7	7.7	7.7	7.7	7.7	7.7	7.7	7.7
24	0	7.0	6.7	7.3	7.3	7.3	7.3	7.3	7.0
41	17	6.7	6.7	7.0	7.0	7.0	7.0	7.0	7.0
47	23	6.4	6.4	7.0	7.0	7.0	7.0	7.0	7.0
65	41	6.4	6.7	7.0	7.0	7.0	7.0	7.0	6.7
71	47	6.7	6.7	7.0	7.0	7.0	7.0	7.0	7.0
89	65	6.7	6.7	6.7	7.0	7.0	7.0	6.7	6.7

5.2.2.3 ProSavin titre® data

Titre data is presented in Figures 5.11 and 5.12. To note, duplicate bioreactor runs performed at the same rocking rate did not yield comparable titres (Figure 5.11). Although titre profiles were similar, as peak titres were observed at approximately the same harvest point (± 6 hr, Figure 5.11), maximum titres values obtained during duplicate runs differed by 1.4 fold (24 rocks min^{-1}), 1.7 fold (18 rocks min^{-1}), 2.2 fold (12 rocks min^{-1}) and 4.6 fold (6 rocks min^{-1}). The fold-differences between duplicate runs performed at 24, 18 and 12 rocks min^{-1} equalled or slightly exceeded those observed for parallel shake flasks (Section 5.2.2.6), perhaps indicating that WAVE cultures are marginally more susceptible to batch-to-batch variation than small scale cultures. The fold-difference between duplicate runs performed at 6 rocks min^{-1} greatly exceeded that observed for parallel shake flasks, suggesting that the sub-optimal culture

conditions prevailing throughout these runs may have exerted an additional pressure that resulted in variable culture performance.

From the mean data, it may be observed that runs performed using a mid-range rocking rate of 12 or 18 rocks min^{-1} typically yielded the highest ProSavin® titres (Figure 5.12). The lower titres observed for runs performed using a rocking of 6 or 24 rock min^{-1} may be attributed to cellular oxygen limitations (leading to increased lactate production and culture acidification) in the former, and exposure of the vector to relatively high levels of turbulence in the latter (this is discussed further in Section 5.3.2). Overall, these results therefore correspond with the earlier microwell observations that adequate aeration combined with gentle fluid mixing is critical for maximising ProSavin® titres (Section 4.3.2).

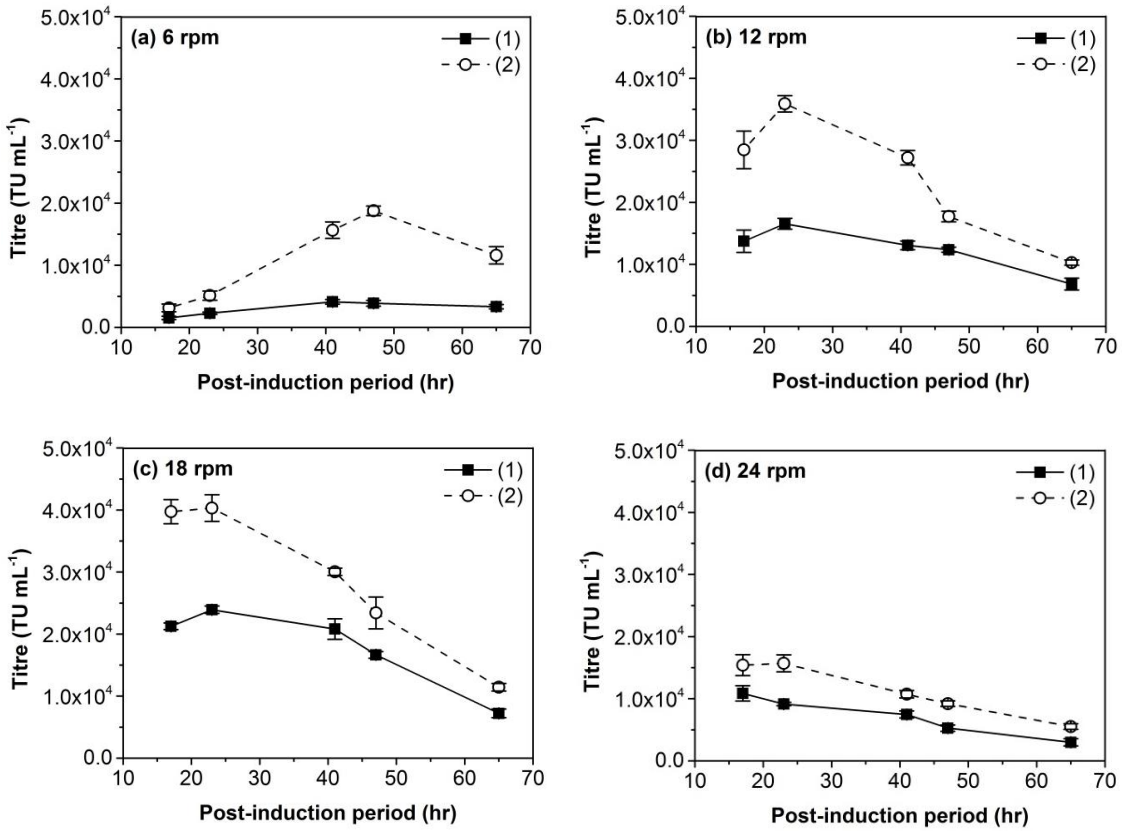


Figure 5.11 ProSavin® titres obtained from PS46.2 cells cultured in a WAVE bioreactor rocked at a rate of (a) 6 rpm, (b) 12 rpm, (c) 18 rpm, or (d) 24 rpm, where 'rpm' is an abbreviation for rocks min^{-1}. Operating conditions were as noted for Figure 5.5. Eight separate bioreactor runs were performed (two at each rocking condition), and data from individual runs is presented (at each time point, sample material was subjected to three repeat measurements, and the mean ± 1 SD is displayed).

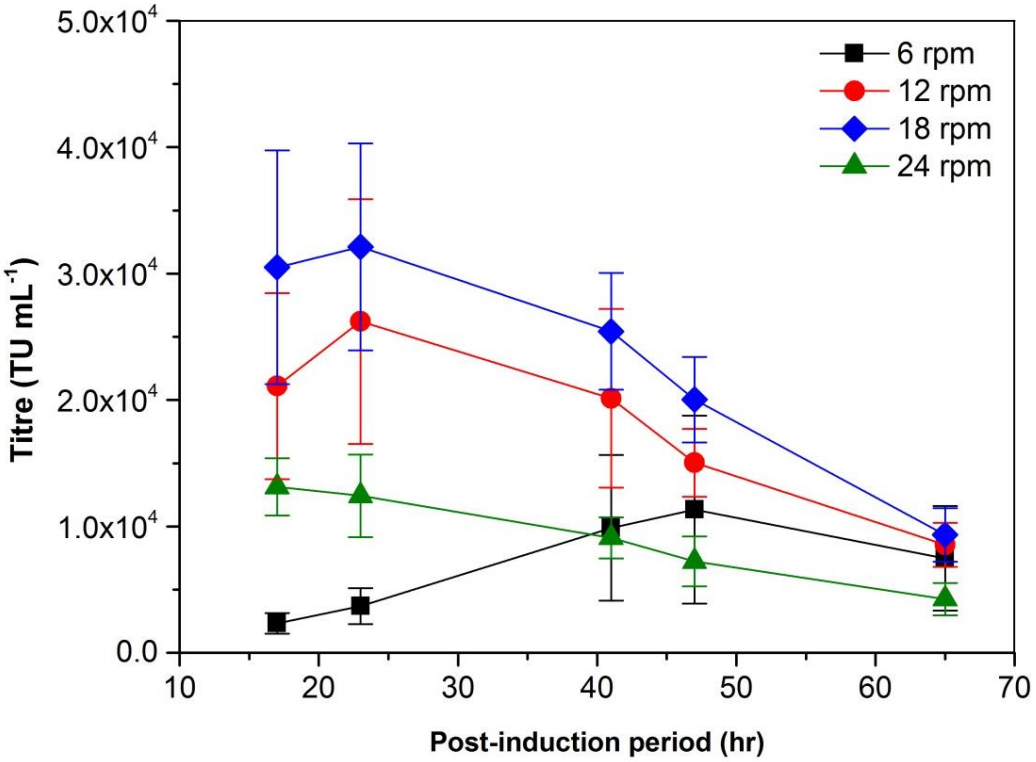


Figure 5.12 Summary graph illustrating ProSavin® titres obtained from PS46.2 cells cultured in a WAVE bioreactor rocked at a rate of 6, 12, 18, or 24 rpm (where 'rpm' is an abbreviation for rocks min^{-1}). Operating conditions were as noted for Figure 5.5. Eight separate bioreactor runs were performed (two at each rocking condition), and mean data from duplicate runs is presented (error bars represent the range).

During the earlier microwell investigations, it was discovered that the optimum time to harvest was within a period spanning approximately 20 hr, which fell between 26 and 46 hr post-induction, although it was noted that this window was likely to vary slightly between batches (Section 4.2.1.5). For the runs performed using agitation conditions most conducive to ProSavin® production (i.e. those performed at 12 or 18 rocks min^{-1}), the highest titres were attained at 23 hr post-induction (Figure 5.12). By 41 hr post-induction, titres had reduced to 75 - 87 % of those observed at 23 hr post-induction. Thus the optimum harvest window occurred slightly earlier during the WAVE bioreactor experiments than was predicted based on the previous (Section 4.2.1.5) microwell data. However, the microwell cultures carried out in parallel to the first two pilot WAVE runs also yielded maximum titres at 22 - 23 hr post-induction, with values declining to 68 - 78 % of the maximum by 40 - 41 hr post-induction (Figures 5.2 and 5.4). This implies that batch-batch variability in the seeding material was most likely responsible

for the shift in the optimum harvest window (the experiments described in this chapter were conducted during a separate time period to the experiments described in Chapters 3 and 4, thus the batch of medium used differed, for example) and as the optimum harvest time was also close to (within 3 hr of) the optimum harvest window predicted during the earlier (Section 4.2.1.5) microwell experiments, it may be concluded that the microwell data provided a good indication of the best time to harvest when using the WAVE system. The maximum titre values obtained were 2.4 - 4.1 x 10^4 TU mL^{-1} during the runs performed at 18 rocks min^{-1}, and 1.8 - 3.7 x 10^4 TU mL^{-1} during the runs performed at 12 rocks min^{-1}. Maximum values obtained during the runs performed at 18 rocks min^{-1} were within two to three-fold of the best titres attained using microwells operating under optimised conditions to date (7.2 x 10^4 TU mL^{-1}; Figure 5.2).

5.2.2.4 Product enhanced reverse transcriptase (PERT) data

To supplement titre data, harvested supernatants were also analysed using the PERT assay, as described in Section 2.11.3.2. This method generates a prediction of biological titre (PERT predicted TU mL^{-1}) based on the amount of reverse transcriptase (RT) enzyme present within a given sample. An assumption is made to correlate the total RT present in a sample to the number of virus particles, however it is not able to predict functionality of the particles. This data is presented in Figures 5.13 and 5.14. Overall there was little variability between duplicate runs. Consistent with biological titre data (Figure 5.11 [a]), the greatest difference between duplicate runs occurred when a rocking rate of 6 rocks min^{-1} was used (Figure 5.13 [a]), with the second run outperforming the first (maximum PERT values differed by 2.2-fold).

PERT predicted titres were affected by rocking rate (Figure 5.14). The results were broadly consistent with biological titre data (Figure 5.12), in that the runs performed at 18 rocks min^{-1} yielded the highest values overall, followed by in descending order, runs performed at 12, 24 and 6 rocks min^{-1}. However, in comparing Figure 5.12 with 5.14 we see that there is a disagreement in the trends presented. Figure 5.12 shows titres reaching a maximum at 17 - 23 hr post induction, with the exception of 6 rocks min^{-1}, and then a general decline in titre. However, Figure 5.14 shows PERT predicted titres increasing over time. The PERT assay overestimated functional titre values by 9- to 192-fold, with the fold-difference generally increasing over time (Table 5.5). This reflects the fact that viral RT associated with ProSavin®

particles is relatively stable and unlikely to be degraded over these production timescales, while functional ProSavin® particles were likely subjected to continuous inactivation (see Chapter 6). The inherent robustness of the RT enzyme makes the PERT assay useful for gaining insight into cellular productivity over time (as the RT is not liable to rapid decay, it presumably builds up in the supernatant at a rate relative to that at which lentiviral vector particles are produced). Here, for those runs performed using a rocking rate of 6 rocks min^{-1}, PERT values started low (relative to all other runs), but increased steadily throughout the duration of the culture (Figure 5.13 [a]). For all other runs, PERT values increased until around 47 hr post-induction, after which they levelled off or increased only marginally (Figure 5.13 [b-d]). Note that maximum biological titre values were also observed later (41 - 47 hr post-induction) during the runs performed at 6 rocks min^{-1} (Figure 5.11 [a]), as compared to all other runs (17 - 23 hr post-induction; Figure 5.11 [b-d]). Overall, the data suggest that lentiviral vector production was inhibited during the early stages of the culture when a rocking rate of 6 rocks min^{-1} was employed, and this led to functional titres peaking later as compared to all other runs. This was likely a consequence of the sub-optimal (i.e. acidic and oxygen poor) conditions experienced by these cultures (Section 5.2.2.2).

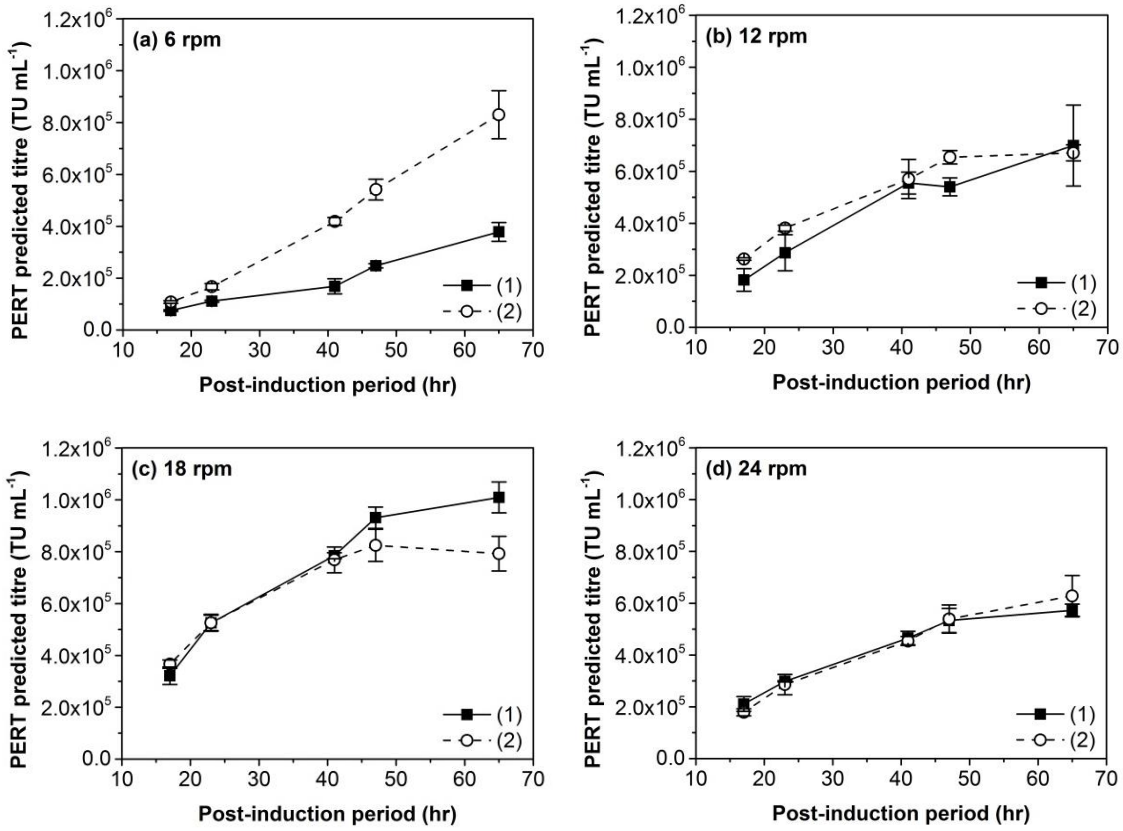


Figure 5.13 Product enhanced reverse transcriptase (PERT) predicted titres obtained during the culture of PS46.2 cells in a WAVE bioreactor rocked at a rate of (a) 6 rpm, (b) 12 rpm, (c) 18 rpm, or (d) 24 rpm, where 'rpm' is an abbreviation for rocks min^{-1}. Operating conditions were as noted for Figure 5.5. Eight separate bioreactor runs were performed (two at each rocking condition), and data from individual runs is presented (at each time point, sample material was subjected to three repeat measurements, and the mean ± 1 SD is displayed).

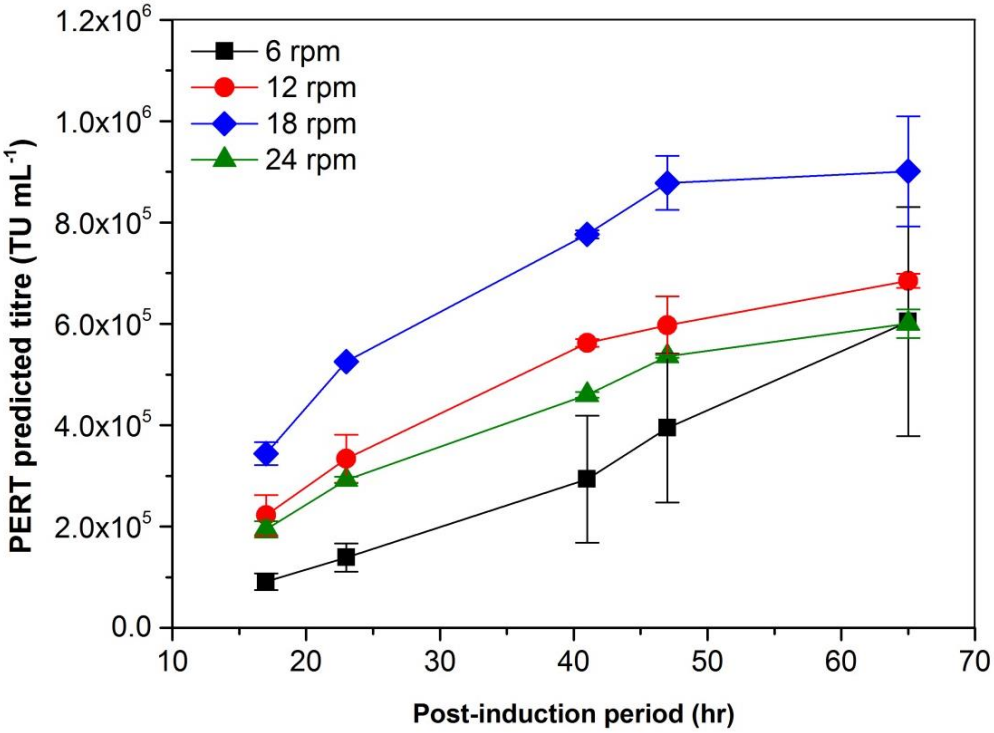


Figure 5.14 Summary graph illustrating PERT predicted titres obtained during the culture of PS46.2 cells in a WAVE bioreactor rocked at a rate of 6, 12, 18, or 24 rpm (where 'rpm' is an abbreviation for rocks min^{-1}). Operating conditions were as noted for Figure 5.5. Eight separate bioreactor runs were performed (two at each rocking condition), and mean data from duplicate runs is presented (error bars represent the range).

Table 5.5 Fold difference between PERT predicted titres and actual biological titres at various time points during the culture of PS46.2 cells in a WAVE bioreactor rocked at a rate of 6, 12, 18, or 24 rpm (where 'rpm' is an abbreviation for rocks min^{-1}). Fold-difference was calculated using mean data values from Figures 5.11 and 5.13 (i.e. fold difference = PERT predicted titre / titre). Operating conditions were as noted for Figure 5.5. Eight separate bioreactor runs were performed (two at each rocking condition), and data from individual runs is presented (n = 1).

Post-induction period (hr)	PERT predicted titre / titre							
	6 rpm		12 rpm		18 rpm		24 rpm	
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
17	49	34	13	9	15	9	19	12
23	49	33	17	11	22	13	33	18
41	41	27	42	21	38	26	62	42
47	64	29	44	37	56	35	101	59
65	113	72	103	65	140	69	192	114

5.2.2.5 RNA copy number data

To supplement ProSavin® titre (Section 5.2.2.3) and PERT predicted titre (Section 5.2.2.4) data, total lentivirus particles were also quantified using the RNA copy number assay. Duplicate WAVE bioreactor runs generated similar RNA copy number profiles (Figure 5.15). Both RNA copy number (Figure 5.15) and PERT (Figure 5.13) data thus appear less susceptible to batch-to-batch variability as compared to functional titre values (Figure 5.11). This is probably because a strand of RNA or a molecule of RT is significantly less complex than a functioning lentiviral vector, thus fewer factors likely play a role in determining their final concentration. Runs performed using a rocking rate of 12, 18 or 24 rock min^{-1} yielded similar RNA copy number profiles, with values typically peaking at 41 - 47 hr post-induction (measuring 2.5 - 4.0 x 10^9 RNA copies mL^{-1}), after which values levelled off or declined marginally (Figure 5.16). Similar to PERT data, those runs performed using a rocking rate of 6 rocks min^{-1} displayed a different profile: RNA copy number values started low (relative to all other runs), but increased steadily for the duration of the culture, reaching 2.1 - 2.5 x 10^9 RNA copies mL^{-1} by 65 hr post-induction (Figure 5.16). The RNA copy number data support the notion that lentiviral vector production was impeded during the early stages of the culture when a rocking rate of 6 rocks min^{-1} was employed.

RNA copy number data was used in conjunction with ProSavin® titre data to calculate P:I ratio values for each time point (P:I ratio = RNA copy number / titre). This data is presented in Table 5.6. As was observed during the earlier microwell optimisation studies (Section 4.2.1) and during the second pilot WAVE study (Section 5.2.1.2), culture supernatants contained an increasing proportion of defective particles over time, and the highest (worst) P:I ratios were observed at the termination of the culture (65 hr post-induction; Table 5.6). The lowest (best) P:I ratio values were attained during the runs performed at 18 rocks min^{-1} and during the second run performed at 12 rocks min^{-1}, where values of 0.4 - 0.5 x 10^5 were recorded at 17 and 23 hr post-induction (Table 5.6), corresponding with the times at which the highest functional titres values were also recorded (Figure 5.11 [b-c]).

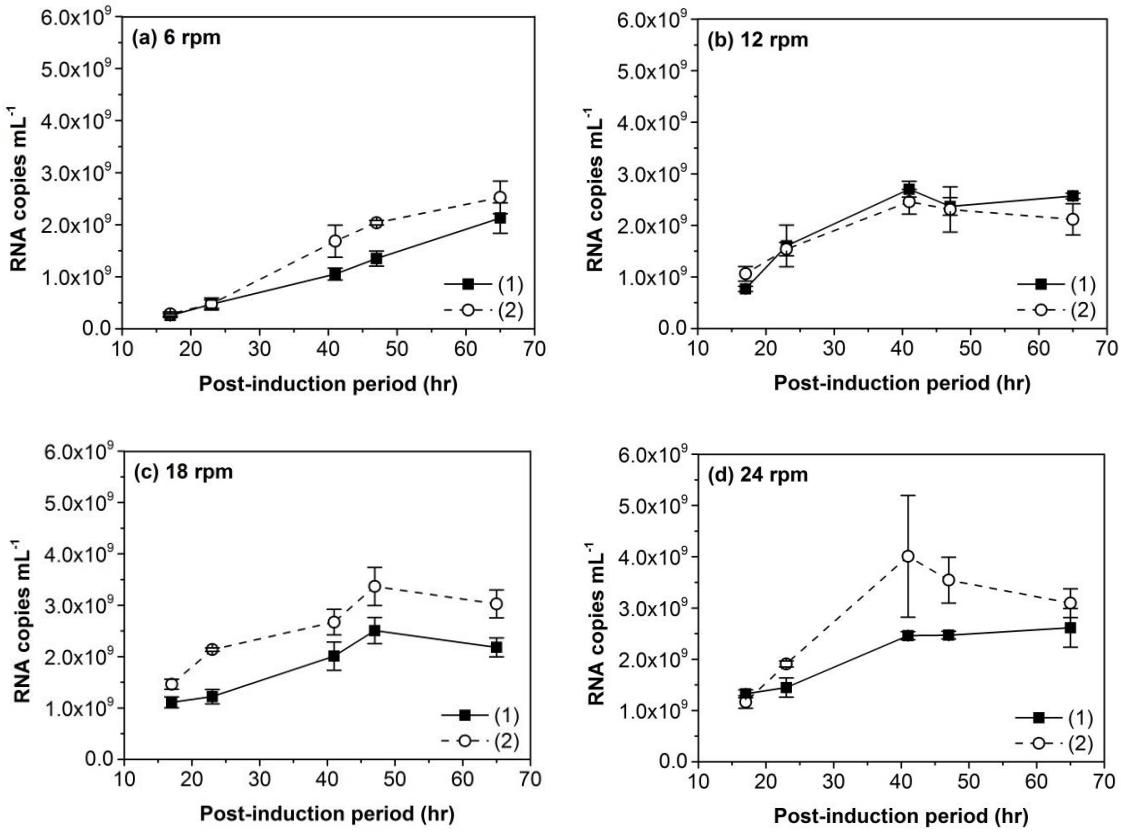


Figure 5.15 RNA copy number data obtained during the culture of PS46.2 cells in a WAVE bioreactor rocked at a rate of (a) 6 rpm, (b) 12 rpm, (c) 18 rpm, or (d) 24 rpm, where 'rpm' is an abbreviation for rocks min^{-1}. Operating conditions were as noted for Figure 5.5. Eight separate bioreactor runs were performed (two at each rocking condition), and data from individual runs is presented (at each time point, sample material was subjected to three repeat measurements, and the mean ± 1 SD is displayed).

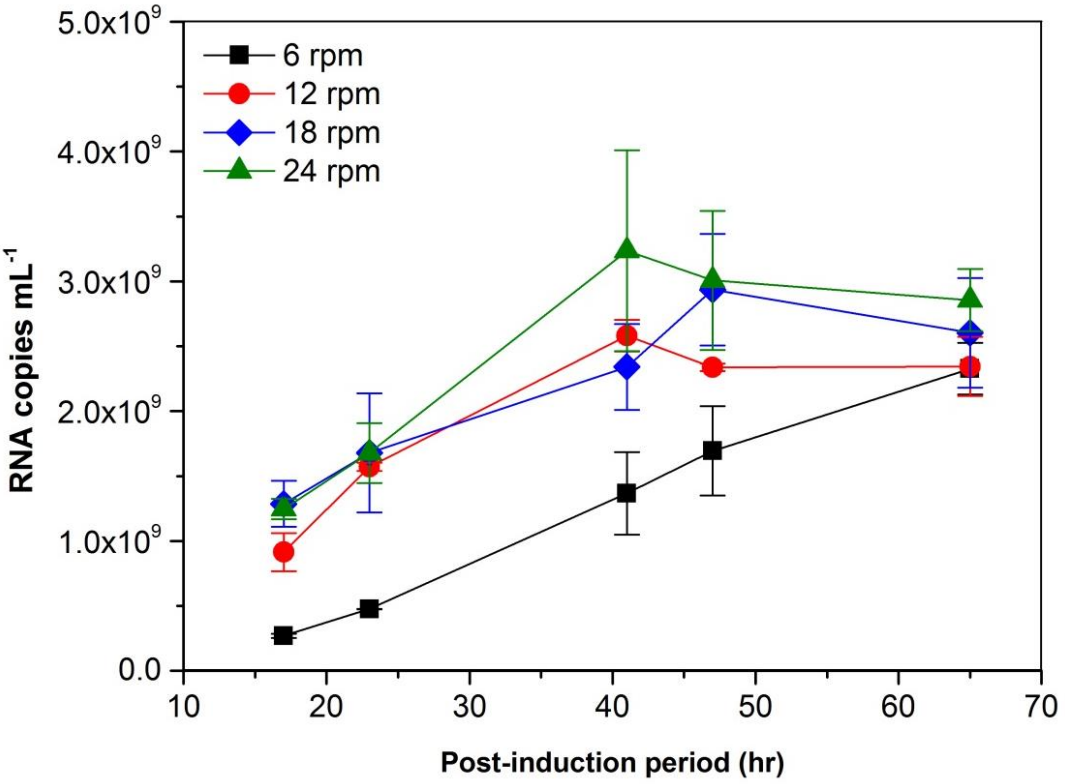


Figure 5.16 Summary graph illustrating RNA copy number data obtained during the culture of PS46.2 cells in a WAVE bioreactor rocked at a rate of 6, 12, 18, or 24 rpm (where 'rpm' is an abbreviation for rocks min^{-1}). Operating conditions were as noted for Figure 5.5. Eight separate bioreactor runs were performed (two at each rocking condition), and mean data from duplicate runs is presented (error bars represent the range).

Table 5.6 Particle:infectivity (P:I) ratios (RNA copy number / titre) at various time points during the culture of PS46.2 cells in a WAVE bioreactor rocked at a rate of 6, 12, 18, or 24 rpm (where 'rpm' is an abbreviation for rocks min^{-1}). Operating conditions were as noted for Figure 5.5. Eight separate bioreactor runs were performed (two at each rocking condition), and data from individual runs is presented (n = 1: values were calculated using mean data from Figures 5.11 and 5.15).

Post-induction period (hr)	P:I ratio (x 10^5)							
	6 rpm		12 rpm		18 rpm		24 rpm	
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
17	1.7	0.9	0.6	0.4	0.5	0.4	1.2	0.8
23	2.1	0.9	1.0	0.4	0.5	0.5	1.6	1.2
41	2.5	1.1	2.1	0.9	1.0	0.9	3.3	3.7
47	3.5	1.1	1.9	1.3	1.5	1.4	4.7	3.8
65	6.4	2.2	3.8	2.1	3.0	2.6	8.8	5.6

5.2.2.6 Capturing experimental variance - Parallel shake flask reference data

Due to equipment limitations, it was not feasible to conduct more than one WAVE bioreactor run at a time. The eight bioreactor runs were therefore conducted on different days. In order to distinguish differences between runs that were caused by the controlled variable from those likely arising due to experimental variance in the process, the following precautions were taken: (i) two independent bioreactor runs were conducted for each rocking condition, (ii) cell growth parameters and lentiviral vector characteristics were quantified three times at each sampling point, (iii) cells used to seed the Cellbag were derived from the same cell bank, and were always used at the same passage number (ten days post-revival), (iv) excepting the difference in rocking rate, exactly the same procedure was followed during each run, and (v) data from reference shake flask cultures was collected in parallel to each run.

Reference shake flask data is presented in Figure 5.17. Triplicate flasks were seeded in parallel to each WAVE bioreactor run and ProSavin® was generated using standard methods (Section 2.4.1.3). Harvested supernatants were then analysed alongside samples from the corresponding WAVE bioreactor run. The main purpose of collecting data from reference shake flask cultures was to monitor process variability, and to help identify the source of any inconsistencies, had they arisen, in the WAVE bioreactor data and thus to ascertain whether a particular cell stock or batch of media used during a particular run could have been responsible for any anomalous results. The data collected from the eight sets of reference shake flasks was relatively consistent, indicating that batch-to-batch differences in the inoculum material used to seed the WAVE bioreactor were small (Figure 5.17). The viable cell concentration of flask cultures (recorded at the time of vector harvest) ranged from 3.2 to 4.5 x 10^6 cells mL^{-1}, while viabilities were between 92 and 95 % (Figure 5.17 [a-b]). Recorded ProSavin® titres were between 3.3 and 5.5 x 10^4 TU mL^{-1}, PERT predicted titres between 0.6 and 1.1 x 10^6 TU mL^{-1}, and RNA copy numbers between 2.0 and 3.8 x 10^9 RNA copies mL^{-1} (Figure 5.17 [c-e]). Although batch-to-batch variances therefore existed, the difference between the highest and lowest recorded values for all measured parameters was less than 2-fold. Also, importantly, the variation between control flasks was random, indicating that the trends observed in the WAVE bioreactor dataset were real and had not been artificially introduced as a consequence of seed stock differences. For example, WAVE runs performed using a rocking rate of 18 rocks min^{-1}

produced consistently higher titres than those conducted using a rocking rate of 24 rocks min^{-1} (Figure 5.11), however this trend was not replicated in control flask titres. Overall, it may be concluded that the use of different cell stocks and media during each WAVE run should not have impacted on the overall findings arising from this dataset (Sections 5.2.2.1 to 5.2.2.5).

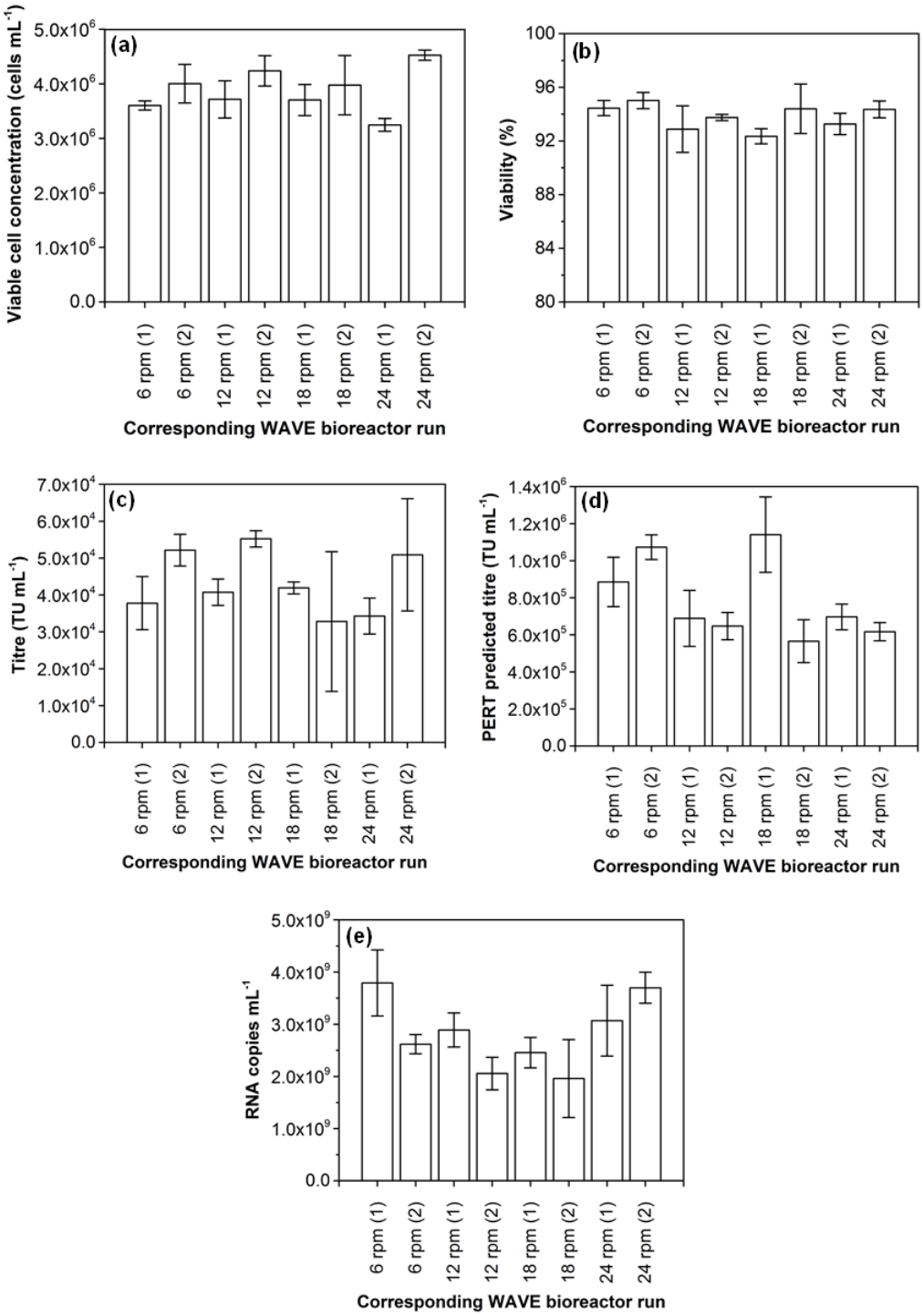


Figure 5.17 Reference shake flask data collected in parallel to WAVE bioreactor runs. (a) Viable cell concentration, (b) cell viability, (c) titre, (d) PERT predicted titre, and (e) RNA copy number data. The corresponding WAVE bioreactor run to which shake flask data relates is indicated by the rocking rate (rpm, an abbreviation for 'rocks min^{-1}') and whether it was the first (1) or second (2) of duplicate runs carried out using those conditions. Triplicate shake flasks were seeded using the same cell stock as was used to seed the WAVE bioreactor. Shake flask operating conditions were as outlined in Section 2.4.1.3. One vector harvest was performed (24 hr post-induction), and cell data (a-b) was collected immediately prior to vector harvests. Mean values are presented and error bars represent ± 1 SD (n = 3).

5.3 Chapter discussion

The development of scalable cell culture processes for the manufacture of lentiviral vectors is an area requiring urgent attention (Ansorge *et al.*, 2010; Schweizer and Merten, 2010; Segura *et al.*, 2013). Here, the insights gained during the earlier microwell investigations (Chapters 3 - 4) were used to rapidly establish operating conditions for ProSavin® production in a 2 L WAVE bioreactor. Although WAVE bioreactors have been previously employed for lentiviral vector production (Throm *et al.*, 2009; Greene *et al.*, 2012; Witting *et al.*, 2012), the protocols described employed either anchorage-dependent tet-OFF producer cells (Throm *et al.*, 2009; Greene *et al.*, 2012) or suspension-adapted cells that required 4 plasmid transient transfection (Witting *et al.*, 2012). Consequently, this chapter comprises the first description of lentiviral vector production in a WAVE bioreactor using a suspension-adapted stable producer (tet-ON) cell line.

5.3.1 Insights obtained at the microwell scale enabled rapid establishment of a protocol for ProSavin® production in a WAVE bioreactor

The first objective of this work was to establish a basic operating procedure for ProSavin® production using a WAVE bioreactor fitted with a 2 L Cellbag. An initial evaluative study, conducted using a 1 L fill volume, yielded an acidic culture environment and low titres (maximum: 8.9×10^3 TU mL^{-1}; Section 5.2.1.1). Earlier microwell data had shown that culture acidification occurs when high fill volumes are used, presumably because this restricts the supply of oxygen to cells, which respond by increasing lactate production. This subsequently accumulates and forces a reduction in culture pH (Sections 3.2.1.2, 3.2.1.3 and 4.2.1.4). A second evaluative study was therefore conducted using a reduced Cellbag fill volume of 0.5 L. The maximum titre value obtained during this run was appreciably higher (2.5×10^4 TU mL^{-1}) and no culture acidification was observed (Section 5.2.1.2). Thus, insights obtained at the microwell scale into the relationship between pH, titre and fill volume enabled rapid establishment of an operating procedure in a WAVE bioreactor that yielded titres within two to three-fold of the maximum titres we have obtained in microwells to date. Note that, although the potential benefit of pre-coating the Cellbag with culture medium was also trialled, this was found to exacerbate cellular aggregation and negatively impact on ProSavin® titres.

5.3.2 The importance of gentle mixing, oxygenation and harvest timing were verified using the WAVE bioreactor

Earlier microwell studies had revealed that liquid fill volume and post-induction period were critical factors influencing ProSavin® production from suspension cultures of PS46.2 (Sections 3.2.5 and 4.2.1). Liquid fill volume affects microwell mixing, and it was demonstrated that low fill volumes (≤ 30 % of total well volume) combined with gentle agitation conditions promoted PS46.2 cell growth and ProSavin® production (Sections 3.2.5, 4.2.1 and 4.2.2). The optimum time to harvest was found to be restricted to within a window spanning a period of around 20 hr (between 26 and 46 hr post-induction, although it was noted this was likely to be batch-specific; Sections 4.2.1.5 and 4.3.1). As further optimisation of these factors in the WAVE bioreactor could lead to improvements in titres, a further set of eight bioreactor runs were performed (Section 5.2.2). As the earlier WAVE bioreactor runs (Section 5.2.1) had demonstrated that a 0.5 L (25 %) fill volume was able to deliver sufficient aeration (oxygen saturation levels remained > 50 % and no culture acidification occurred), this factor remained fixed, while the rocking rate was varied (6, 12, 18 and 24 rock min^{-1}; n = 2) to generate different mixing regimes, and multiple harvests were performed. By monitoring various cell culture parameters (cell growth, dissolved oxygen and pH) and lentiviral vector characteristics (ProSavin® [functional] titres, PERT predicted titres and RNA copy numbers), it was possible to gain insight into how these responses both interacted and reacted to changes in rocking rate.

Overall, the runs performed using rocking rate of 18 rocks min^{-1}, closely followed by those performed using a rocking rate of 12 rocks min^{-1}, yielded the highest titres and best P:I ratios (Sections 5.2.2.3 and 5.2.2.5). The relatively low titres (≤ 1.9×10^4 TU mL^{-1}) observed during the runs conducted at 6 rocks min^{-1} were attributed to the sub-optimal conditions experienced by these cultures as a result of inadequate mixing. During these runs, oxygen levels were consistently lower than the 50 % saturation value thought optimal for maintaining mammalian cell growth (Miller *et al.*, 1987), and significant acidification of the culture (to pH 6.4) occurred (Section 5.2.2.2). The culture acidification that occurred during the 6 rocks min^{-1} run likely reduced the productivity of PS46.2 cells, as well as increased the rate of ProSavin® inactivation, as lentiviral vectors have been reported to decay at a faster rate when subjected to acidic (as compared to pH neutral) conditions (Higashikawa and Chang, 2001). During all other runs,

dissolved oxygen levels remained above 50 %, and the culture pH stabilised at around pH 7. Interestingly, cell growth did not appear inhibited during the runs performed at 6 rocks min^{-1} as cell viabilities were consistently high, above 85 %; for all other rocking rates, cell viabilities dropped to below 75 % (Section 5.2.2.1). The cell viability was perhaps preserved during the runs conducted at 6 rock min^{-1} because exposure to hydrodynamic stress (Eibl *et al.*, 2010b) and oxidative damage (Miller *et al.*, 1987) was reduced. Although a rocking rate of 6 rocks min^{-1} was not damaging to cell growth or survival, it was detrimental for lentiviral vector production. PERT predicted titres and RNA copy numbers were initially (i.e. when measured at 17 hr post-induction) lowest during the runs conducted at 6 rock min^{-1} as compared to all other runs, however, their levels steadily increased throughout the culture (during all other runs levels stabilised after 47 hr post-induction; Sections 5.2.2.4 and 5.2.2.5). Functional titres also peaked later (41 - 47 hr post-induction) during the runs performed at 6 rocks min^{-1} (in all other runs maximum titres were obtained 17 - 23 hr post-induction; Section 5.2.2.3). Taken together, the data indicates that lentiviral vector production was inhibited during the early stages of the culture when a rocking rate of 6 rocks min^{-1} was used. It seems that, although the cells are able to survive on relatively little oxygen (Figure 5.10: 6 rocks min^{-1} produced the least oxygen blending), the production of lentiviruses requires an increased supply of oxygen that a rocking rate of 6 rocks min^{-1} could not deliver. Titres were also relatively low ($\leq 1.6 \times 10^4$ TU mL^{-1}) when the rocking rate was increased to 24 rocks min^{-1}. These cultures experienced consistently high dissolved oxygen levels (> 80 %; Section 5.2.2.2), but otherwise performed similarly to the 12 and 18 rocks min^{-1} cultures. Of the rocking rates examined, a rocking rate of 24 rocks min^{-1} likely produced the most turbulent conditions (Eibl and Eibl, 2006; Eibl *et al.*, 2010b), and it is possible that this environment accelerated the rate of vector inactivation: the low titres obtained during these runs were not accompanied by reduced PERT and RNA copy number values, which suggests that the stability, rather than the production, of vector particles may have been compromised. Overall, it may be concluded that a mid-range rocking rate (12 - 18 rocks min^{-1}) is optimal for ProSavin® production. A rocking rate of 18 rocks min^{-1} produced consistently high titres (maximum: 2.4 - 4.1 x 10^4 TU mL^{-1}), while a rocking rate of 12 rocks min^{-1} produced more varied results (maximum titres: 1.8 - 3.7 x 10^4 TU mL^{-1}). The P:I ratio at the time at which maximum titres were obtained was 5 x 10^4 during the runs performed at 18 rocks min^{-1}, which is around half of that previously observed in microwells (e.g. Section 4.2.1.5). This is most likely a

direct consequence of titres peaking earlier in the WAVE cultures as defective particles would have had less time to accumulate. Although no real improvement in titre was accomplished as a result of these investigations since maximum titre values were similar to those attained during the earlier pilot run (Section 5.2.1.2), a greater understanding of the operational design space has been achieved.

The objective of these studies was ultimately to verify whether the conclusions generated using the microwell system held true following scale-up of the process. The observation that titres were compromised when cultures experienced either vigorous agitation (i.e. when a rocking rate of 24 rocks min^{-1} was used) or limited oxygen availability (i.e. when a rocking rate of 6 rocks min^{-1} was used), supports the notion that a combination of gentle agitation and adequate oxygenation is important for maximising titres. Evidence that the microwell system correctly predicted the optimum time at which to perform harvests was given by the observation that maximum titres were achieved at 23 hr post induction (during the 12 or 18 rocks min^{-1} runs), which was within 3 hr of the optimum harvest window predicted during the earlier (Section 4.2.1.5) microwell experiments, but precisely matched the microwell data collected in parallel to the first two pilot WAVE runs (Figures 5.2 and 5.4). Overall, the microwell investigations correctly predicted the important parameters affecting ProSavin® titres in WAVE bioreactor cultures, and consequently the microwell platform may be usefully applied to the future development of lentiviral vector processes.

5.3.3 Concluding remarks

In summary, the potential of wave-mixed bioreactor technology for future applications to large-scale lentiviral vector production based on suspension-adapted producer cells has been demonstrated. The bioprocess design data generated using the microscale experimental platform was fundamental in aiding the rapid establishment of operating conditions for ProSavin® production in the WAVE bioreactor. In particular, post-induction period and liquid fill volume were key factors affecting ProSavin® titres at the microwell scale, and optimisation of these factors proved crucial in the development of the larger scale process. The microwell system can therefore be considered a valuable bioprocess development tool.

The WAVE bioreactor was simple to set-up, and the procedure for ProSavin® production straight-forward. As maximum titres were observed at 23 hr post-induction when a rocking rate of 12 or 18 rocks min^{-1} was used, the entire process, from cell inoculation to lentiviral vector harvest, could theoretically be accomplished in three days. As the culture vessel is disposable, no down-time between runs is required. In addition, scale-up to larger vessels should be readily achievable (Eibl *et al.*, 2010b).

Finally, it is evident that titres do not accumulate over time (Section 5.2.2.3) and that functional ProSavin® may have a short half-life at the temperature at which cell culture is performed. It is conceivable that higher titres may be achieved if a perfusion system were developed, as this would allow vector particles to be continually separated from cells via filtration and stored at 4 °C, potentially preventing the rapid loss of functionality seen at these higher temperatures. In addition, the dilution of cytotoxic vector proteins (VSV-G) and metabolic by-products, such as lactate; and the addition of fresh nutrients, could extend cellular productivity. With a view to informing the design of enhanced large-scale processes for lentiviral vectors, the next chapter examines the rate at which ProSavin® and other lentiviral vectors are inactivated and tests approaches to slow the rate of decay.

6 Evaluation of lentiviral vector stability during upstream processing

6.1 Introduction and aims

Throughout the earlier microwell (Chapters 3 and 4) and WAVE bioreactor (Chapter 5) investigations, post-induction period (i.e. harvest timing) was consistently found to be a critical factor impacting on ProSavin® titres. Titres increased following the induction of ProSavin® producer cells (PS46.2), then plateaued and declined, and in three days the optimum harvest window had passed (Figure 4.8, Figure 5.12). The early data suggested that functional ProSavin® is inactivated in culture supernatants, and during the latter stages of batch cultivations the rate of decay either surpasses the rate of production or the rate of production declines. HIV-1 based lentiviral vectors have been reported to exhibit a half-life of between 0.8 and 10.4 hr at 37 °C (Higashikawa and Chang, 2001; Zhang *et al.*, 2004; Carmo *et al.*, 2009a; Carmo *et al.*, 2009b), however the half-life of EIAV-based lentiviral vectors at production temperature was not known. This information could aid the design of improved manufacturing processes for EIAV-based lentiviral vectors. Thus, the overall aim of this chapter was to investigate the rate at which ProSavin® and other EIAV-based lentiviral vectors are inactivated, plus test approaches to moderate the rate of decay. The specific objectives of this chapter are:

- To characterise the stability of functional ProSavin®, vector RNA and reverse transcriptase (RT) under typical production conditions (36.5 °C)
- To propose (based on a review of relevant literature) and test approaches for mitigating ProSavin® inactivation during upstream processing
- To investigate whether the stability of EIAV-based lentiviral vectors is a function of the particular production method or transgene employed

Results included in Section 6.2.1.1 of this chapter were published in: Guy, H. M., *et al*. (2013). *Human Gene Therapy Methods*, **24(2)**: 125-139. Permission to reproduce this content has been granted by Mary Ann Liebert, Inc.

6.2 Results

6.2.1 Characterisation of ProSavin® stability under typical production conditions

6.2.1.1 Stability of ProSavin® in crude supernatants at 36.5 °C

The stability of functional ProSavin®, vector RNA and RT in cell culture supernatants incubated at 36.5 °C was characterised. In the first experiment, supernatants harvested from standard shake flask cultures of PS46.2 were aliquoted into shaken microwells, static microwells or static vials and incubated for various time periods of up to 168 hr (Section 2.8.1). ProSavin® titres were subsequently quantified and this data is presented in Figure 6.1. Similar decay kinetics were observed for all vessels. As the data obtained from the static vials was representative of that obtained when using the microscale culture system, future stability studies were consequently conducted using this format.

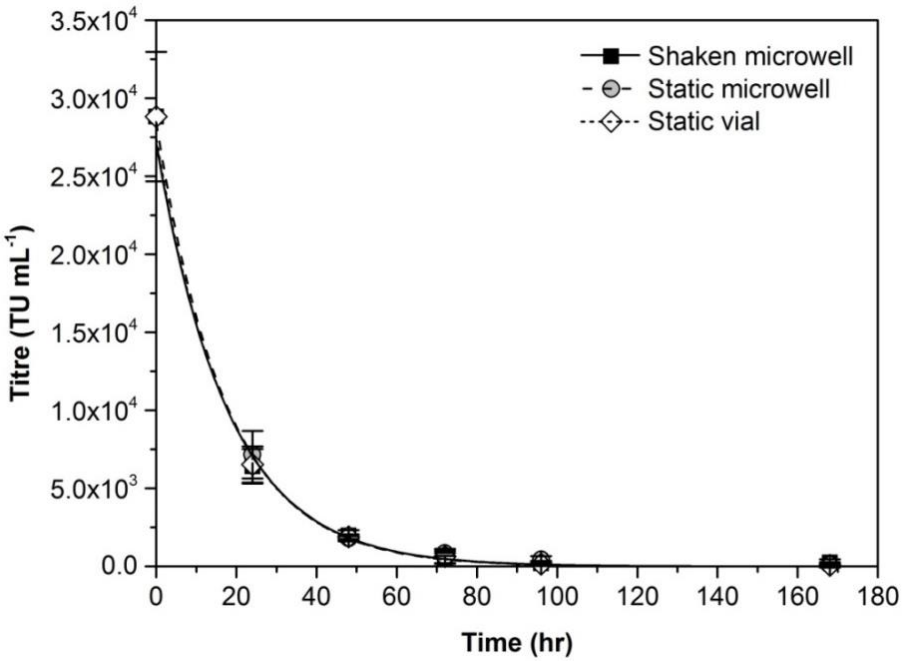
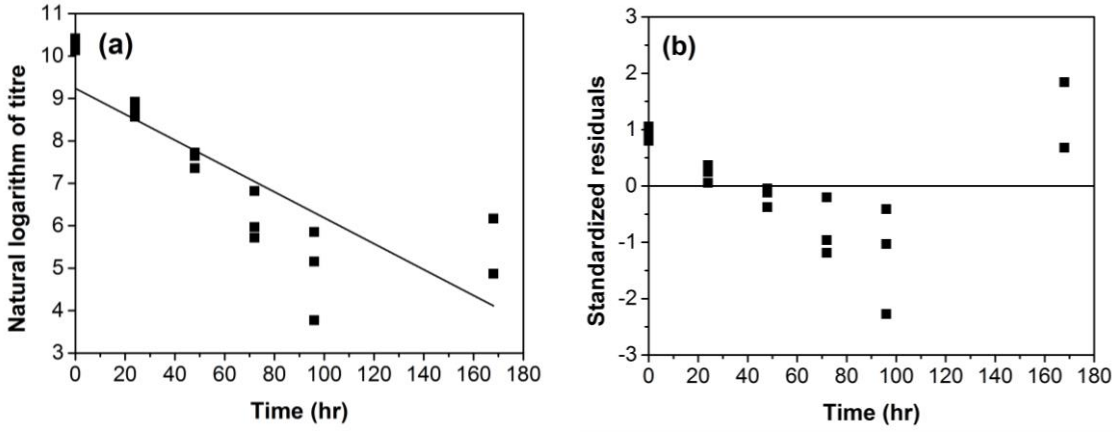


Figure 6.1 Functional ProSavin® stability profiles during the incubation of crude supernatants at 36.5 °C in shaken microwells, static microwells and static vials. Titres were quantified using the rapid ProSavin® titre assay (Section 2.11.2.1). The experimental procedure was as described in Section 2.8.1. Mean data is presented and error bars represent ± 1 SD (n = 3). Exponential trend lines were fitted according to the results of linear regression analysis (presented in Table 6.2): to construct trend lines, the slope value [*b*] and the exponent of the intercept value [*a*] were entered into the equation $y = ae^{bx}$.

To calculate the half-life ($t_{½}$) of functional ProSavin®, titre data was transformed by taking the natural logarithm and analysed using linear regression. The slope value (*b*) was then entered into the following equation (Bryan *et al.*, 1990):

$$t_{\frac{1}{2}} = \frac{-\ln(2)}{b} \quad (6.1)$$

Half-life confidence intervals were calculated using the 'Slopest' method as described by Bryan *et al.* (1990), where *b* (in Equation 6.1) was replaced with the upper and lower limits of the confidence interval for the slope. All titre data collected between 0 - 168 hr was initially included in linear regression analyses. However, for all datasets (shaken microwell, static microwell, and static vial) this resulted in poor model fit. As a representative example, results from the analysis of shaken microwell data are presented in Figure 6.2. The coefficient of determination (R^2) value was 0.713, and the linear fit and residual plots indicated that a linear model inadequately described the change in (log transformed) titre values over time.



(c)

Intercept (*a*)	Slope (*b*)	R^2	*p* value	$t_{½}$ (hr)
9.24 ± 0.89	-0.0305 ± 0.0103	0.713	< 0.0001	22.7 (17.0, 34.3)

Figure 6.2 Results of linear regression analysis of the shaken microwell dataset. Titre data (quantified according to Section 2.11.2.1) was transformed prior to analysis by taking the natural logarithm. Plots illustrate the line of linear fit (a) and the standardized residuals (b). A random scattering of data points about the horizontal (zero) axis of the standardized residual plot (*not* as observed here) is indicative of a well-fitting linear model. The statistical output of the linear regression is also provided (c). Slope and intercept values include a 95 % confidence interval. A *p* value < 0.05 indicates that the slope is significantly different from zero. The half-life of functional ProSavin® (with upper and lower 95 % confidence limits) was calculated using Equation 6.1 as described earlier.

As the majority (93 - 94 %) of functional ProSavin® was inactivated by 48 hr, titres values obtained after this point, at 72, 96 and 168 hr, were exceptionally low (≤ 8.4 x 10^2 TU mL^{-1}; Table 6.1), and approached the limit of detection of the assay. It was therefore considered whether titre values obtained during the latter stages of the incubation period should be excluded from linear regression analyses. It was evident that by 72 hr titres approached levels at which the precision of the ProSavin® assay (which is based on flow cytometry; see Section 2.11.2.1) was compromised, as coefficient of variation (CV) values associated with these samples equalled or significantly exceeded 20 % (Table 6.1). To determine a threshold above which titre values may be considered accurate and consequently suitable for inclusion in linear regression analyses, a dilution series of lentiviral vector samples was prepared and quantified to enable the establishment of a lower limit of quantification (LOQ) for the ProSavin® assay. This work is described in Appendix B. It was demonstrated that when a standard population size of 10,000 cells is analysed using flow cytometry, accurate quantitation may be expected when the

proportion of transduced cells is equal to or greater than around 0.2 %, and consequently this value was employed as the LOQ for all stability studies described in this chapter.

Table 6.1 ProSavin® titres (quantified according to Section 2.11.2.1) during the incubation of crude supernatants at 36.5 °C in shaken microwells, static microwells and static vials. The experimental procedure was as described in Section 2.8.1. Coefficient of variation (CV) values represent the ratio of the standard deviation (SD) to the mean (CV = [SD / mean] * 100).

Time (hr)	Shaken microwell			Static microwell			Static vial		
	Mean titre (TU mL^{-1})	1 SD	CV (%)	Mean titre (TU mL^{-1})	1 SD	CV (%)	Mean titre (TU mL^{-1})	1 SD	CV (%)
0	$2.9x10^4$	$4.2x10^3$	14	$2.9x10^4$	$4.2x10^3$	14	$2.9x10^4$	$4.2x10^3$	14
24	$6.4x10^3$	$1.1x10^3$	17	$7.2x10^3$	$1.5x10^3$	21	$6.5x10^3$	$1.2x10^3$	18
48	$2.0x10^3$	$3.6x10^2$	18	$1.8x10^3$	$1.3x10^2$	7	$1.9x10^3$	$1.5x10^2$	8
72	$5.4x10^2$	$3.3x10^2$	61	$8.4x10^2$	$1.6x10^2$	20	$6.4x10^2$	$4.8x10^2$	75
96	$1.9x10^2$	$1.5x10^2$	81	$4.3x10^2$	$2.2x10^2$	50	$1.5x10^2$	$1.1x10^2$	76
168	$2.5x10^2$	$2.0x10^2$	82	$1.0x10^2$	$1.8x10^2$	173	$7.2x10^1$	$9.0x10^1$	125

With regard to the ProSavin® titre data obtained during the stability experiment (Figure 6.1), from 72 hr onwards at least one replicate per time point per condition fell below the LOQ of the assay. Taken together with the observation of high CV values at these time points, it was concluded that these samples had not been accurately quantified, and thus linear regression analyses were re-performed with these points excluded. For all datasets (shaken microwell, static microwell, static vial), this markedly improved the fit of the linear model. As an example, linear fit and residual plots generated following the analysis of shaken microwell data are shown in Figure 6.3. Statistical outputs from all datasets are included in Table 6.2. In all cases the R^2 value exceeded 0.97. The average half-life of functional ProSavin® in crude supernatants at 36.5 °C was calculated to be 12.2 ± 0.2 hr, regardless of vessel. As so many data points had been excluded from these analyses, however, it was desirable to gather more information at earlier time points to verify these results. In particular, it was noted that the distribution of standardized residuals appeared to curve slightly as a function of time (Figure 6.3 [b]), suggesting that the linear model may yet be inappropriate.

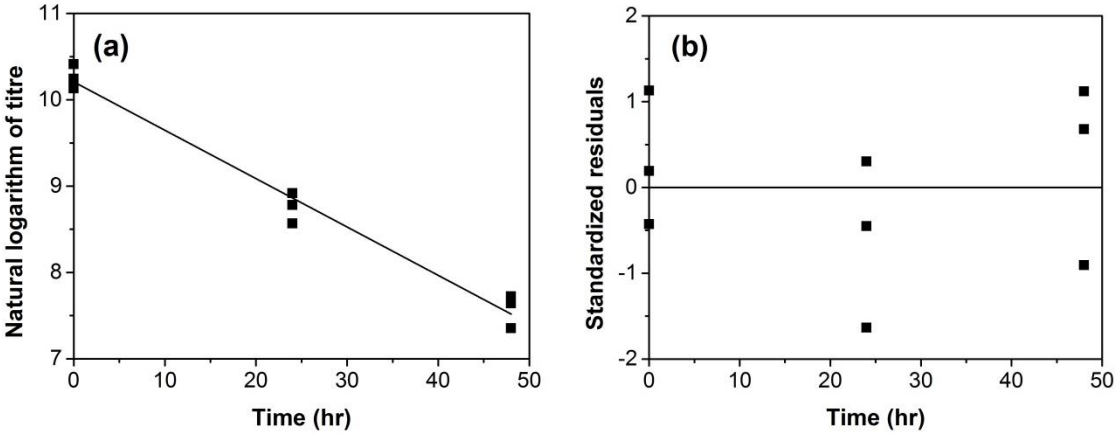


Figure 6.3 Scatter plots illustrating the line of linear fit (a) and the standardized residuals (b) obtained following linear regression analysis of the shaken microwell dataset. Only values obtained during the first 48 hr of incubation were included in analyses. Titre data (quantified according to Section 2.11.2.1) was transformed prior to analysis by taking the natural logarithm.

Table 6.2 Results of linear regression analysis of the shaken microwell, static microwell and static vial datasets. Only values obtained during the first 48 hr of incubation were included in analyses. Titre data (quantified according to Section 2.11.2.1) was transformed prior to analysis by taking the natural logarithm. Slope and intercept values include a 95 % confidence interval. The half-lives ($t_{½}$) of functional ProSavin® (with upper and lower 95 % confidence limits) were calculated using Equation 6.1 as described earlier.

Condition	Intercept (*a*)	Slope (*b*)	R^2	*p* value	$t_{½}$ (hr)
Shaken microwell	10.21 ± 0.23	-0.0560 ± 0.0073	0.979	< 0.0001	12.4 (11.0, 14.2)
Static microwell	10.26 ± 0.19	-0.0577 ± 0.0060	0.987	< 0.0001	12.0 (10.9, 13.4)
Static vial	10.22 ± 0.18	-0.0566 ± 0.0059	0.987	< 0.0001	12.4 (11.1, 13.7)

To better understand the inactivation profile of ProSavin® at 36.5°C, a second experiment was conducted in which a greater number of time points were examined over a shorter period. Supernatants harvested from standard shake flask cultures of PS46.2 were aliquoted into vials and incubated for various time periods of up to 48 hr (Section 2.8.1). ProSavin® titres were subsequently quantified, and this data is presented in Figure 6.4. Linear regression analysis of natural log transformed data returned an average half-life value of 12.0 hr (Figure 6.5 [c]), in agreement with the earlier data (Table 6.2). Despite a reasonable R^2 value (0.957), however, the distribution of standardized residuals appeared to curve as a function of time (Figure 6.5 [b]), similar to as noted previously (Figure 6.3 [b]). Further exploration of the data revealed that

removal of the data obtained at the last two time points (36 and 48 hr) improved the linear fit: the curvature was eradicated from residual data, and the R^2 improved slightly to 0.973 (Figure 6.5 [e] and [f]). Following refinement of the linear model, the average half-life of ProSavin® was calculated to be 10.1 hr during a 0 - 30 hr incubation period (Figure 6.5 [f]). As the 36 and 48 hr data points were within the quantifiable range of the ProSavin® assay, the data indicate that the ProSavin® inactivation profile may be biphasic, and that the average rate of decay is slower after 36 hr.

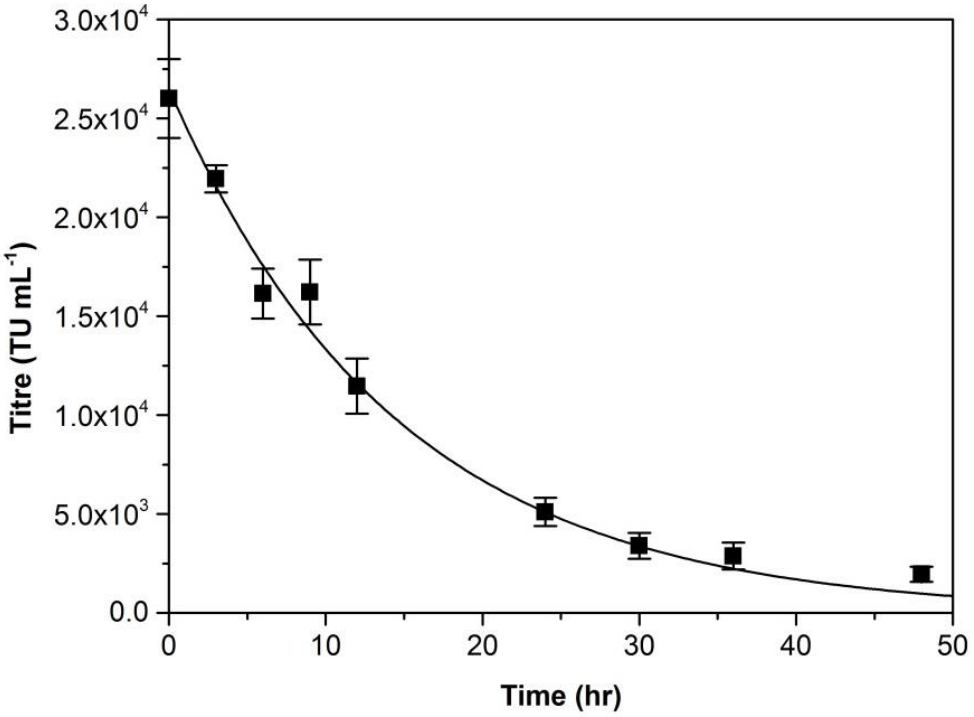
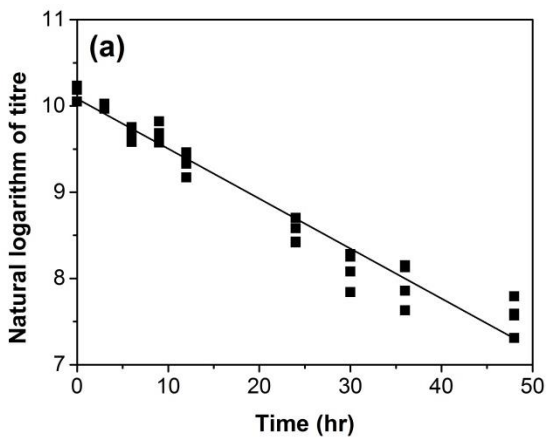
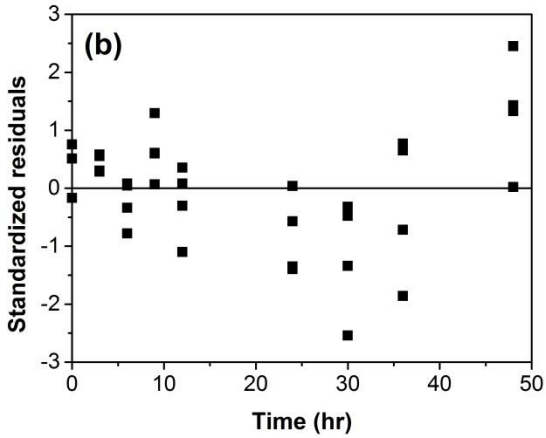


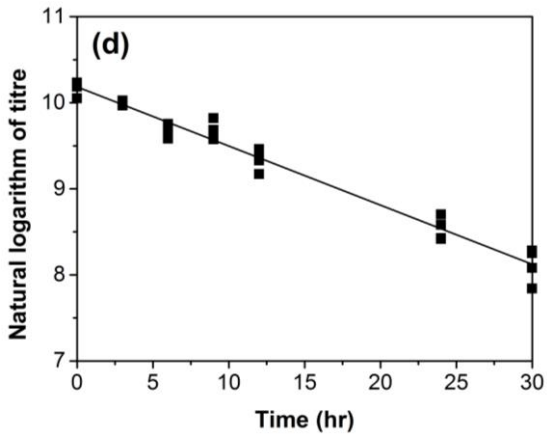
Figure 6.4 Functional ProSavin® stability profile during the incubation of crude supernatant at 36.5 °C. Titres were quantified using the rapid ProSavin® titre assay (Section 2.11.2.1). The experimental procedure was as described in Section 2.8.1. Mean data is presented and error bars represent ± 1 SD (n = 4). The exponential trend line was fitted according to the results of linear regression analysis presented in Figure 6.5 (f). Trend line construction was completed as detailed for Figure 6.1.

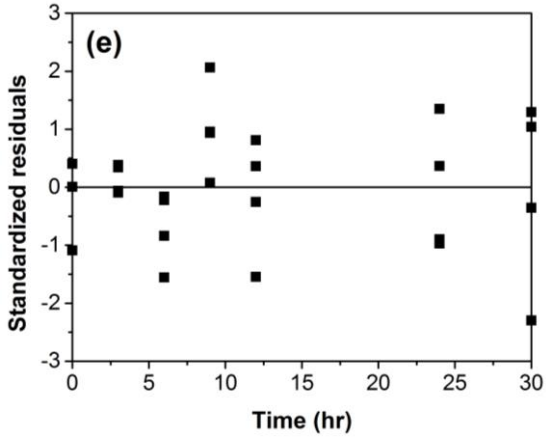




(c)

Intercept (*a*)	Slope (*b*)	R^2	*p* value	$t_{½}$ (hr)
10.08 ± 0.10	-0.0579 ± 0.0043	0.957	< 0.0001	12.0 (11.1, 12.9)



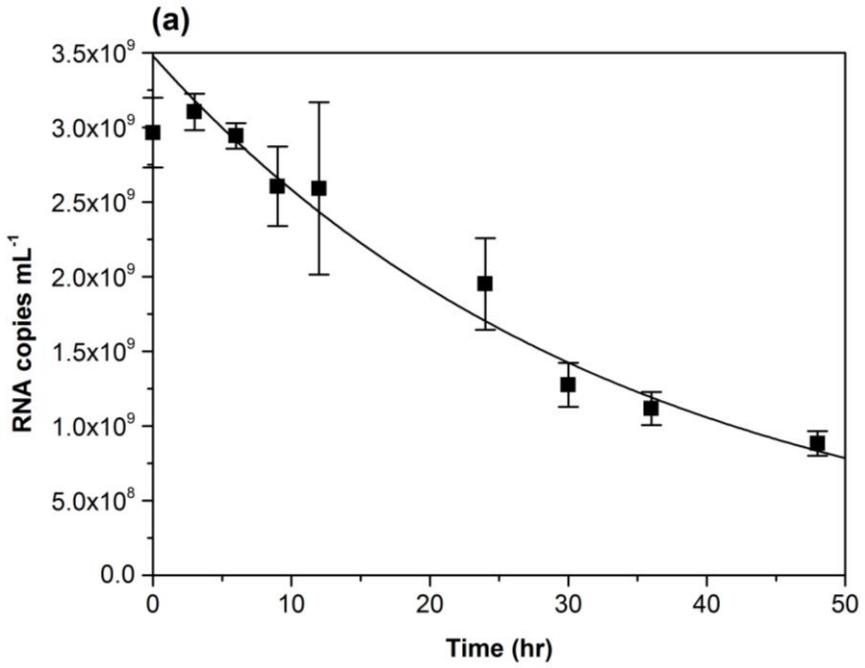


(f)

Intercept (*a*)	Slope (*b*)	R^2	*p* value	$t_{½}$ (hr)
10.18 ± 0.07	-0.0687 ± 0.0047	0.973	< 0.0001	10.1 (9.4, 10.8)

Figure 6.5 Linear regression analysis of ProSavin® titres (quantified according to Section 2.11.2.1) obtained during the incubation of crude supernatant at 36.5 °C (see Figure 6.4). Titre values were transformed by taking the natural logarithm prior to analysis. Plots obtained following analysis of all data points (0 - 48 hr) illustrate the line of linear fit (a) and the standardized residuals (b), while the statistical output of the linear regression is also provided (c). (d), (e) and (f) are the corresponding results obtained following model refinement (where the 36 and 48 hr data points were excluded). Slope and intercept values include a 95 % confidence interval. The half-life of functional ProSavin® (with upper and lower 95 % confidence limits) was calculated using Equation 6.1 as described earlier.

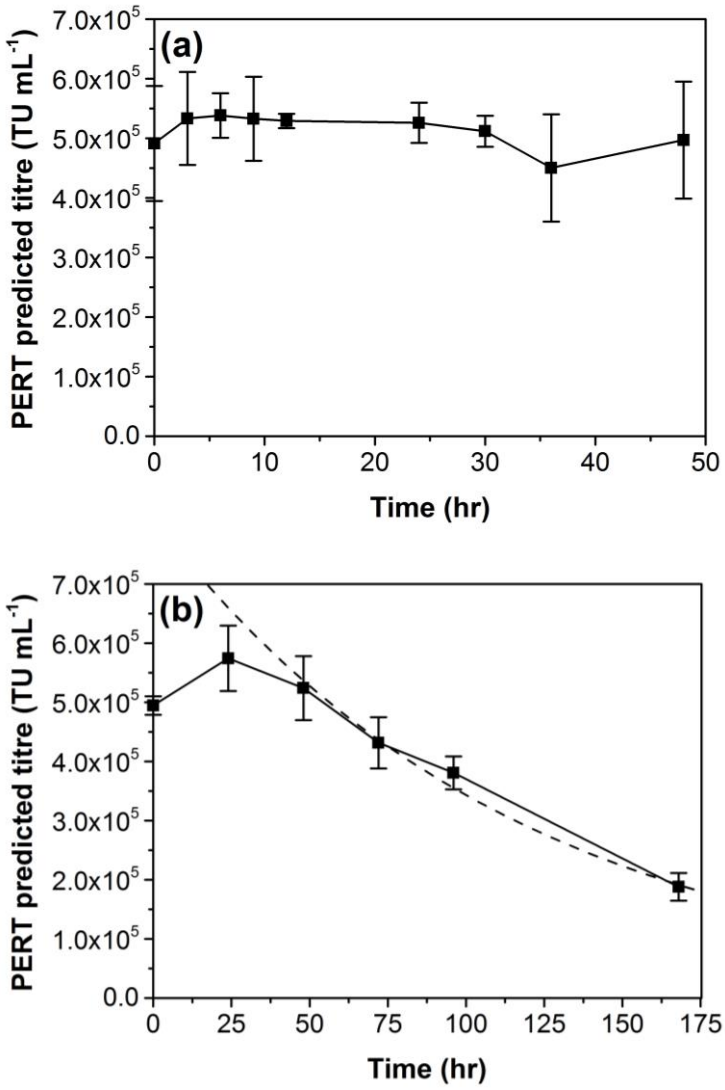
To gain greater insight into the mechanism underlying ProSavin® inactivation at 36.5 °C, vector RNA and reverse transcriptase (RT) activity were also quantified. RNA copy number data is presented in Figure 6.6. The average half-life of vector RNA was calculated to be 23.3 hr over the incubation period 3 - 48 hr. As functional ProSavin® displayed a half-life that was much shorter than this (10.1 hr; Figure 6.5 [f]), it is therefore unlikely that vector RNA degradation is the primary mechanism for ProSavin® inactivation. The PERT assay measures the amount of RT enzyme present within a given sample and uses this information to generate a prediction of biological titre (Section 2.11.3.2). PERT data is presented in Figure 6.7. Over the time period examined in this study (0 - 48 hr) PERT predicted titres did not decline (Figure 6.7 [a]). For interest, samples obtained during the earlier 0 - 168 hr ProSavin® stability study (for which biological titre data is displayed in Figure 6.1) were also analysed using the PERT assay (Figure 6.7 [b]). In agreement with Figure 6.7 [a], PERT predicted titres remained stable during the first 48 hr of incubation (Figure 6.7 [b]), however did subsequently decline. An approximate linear model was fitted to natural log transformed data obtained between 48 and 168 hr incubation, and the average half-life of vector RT was calculated to be 80.6 hr over this period (Figure 6.7 [c]). Overall, it may be concluded that degradation of the RT enzyme is unlikely to contribute to ProSavin® inactivation (the majority [> 90 %] of functional particles were inactivated within 48 hr incubation [Figure 6.1; Figure 6.4] over which period the amount of vector RT associated with ProSavin® particles did not decline [Figure 6.7]).



(b)

Intercept (*a*)	Slope (*b*)	R^2	*p* value	$t_{½}$ (hr)
21.97 ± 0.08	-0.0298 ± 0.0032	0.921	< 0.0001	23.3 (21.0, 26.1)

Figure 6.6 (a) Vector RNA stability profile during the incubation of crude supernatant at 36.5 °C, and (b) results of the corresponding linear regression. The experimental procedure was as described in Section 2.8.1. Mean data is presented in plot (a) and error bars represent ± 1 SD (n = 4). Exponential trend line construction was completed using the method detailed in Figure 6.1, using the results presented in table (b). Note that the 0 hr data points were excluded from linear regression analysis to obtain the best model fit. Values were transformed by taking the natural logarithm prior to linear regression analysis. Slope and intercept values include a 95 % confidence interval. The half-life of vector RNA (with upper and lower 95 % confidence limits) was calculated using Equation 6.1 as described earlier.



(c)

Intercept (*a*)	Slope (*b*)	R^2	*p* value	$t_{½}$ (hr)
13.61 ± 0.15	-0.0086 ± 0.0014	0.947	< 0.0001	80.6 (69.3, 96.3)

Figure 6.7 Vector reverse transcriptase (RT) stability profiles during the incubation of crude supernatant at 36.5 °C. (a) Results following 0 - 48 hr incubation (second experiment) and (b) results following 0 - 168 hr incubation (first experiment; static vial dataset). The statistical output of the linear regression fitted to 48 - 168 hr data (obtained during the first experiment) is also provided (c). Experimental procedures were as described in Section 2.8.1. In plots (a) and (b) mean data is presented and error bars represent ± 1 SD (n = 4 [a] or n = 3 [b]). Construction of the exponential trend line (represented by a dashed line in plot [b]) was completed using the method detailed in Figure 6.1, using the results presented in table (c). PERT predicted titre values were transformed by taking the natural logarithm prior to analysis by linear regression. Slope and intercept values include a 95 % confidence interval. The half-life of vector RT (with upper and lower 95 % confidence limits) was calculated using Equation 6.1 as described earlier.

6.2.1.2 Investigation into whether autotransduction of producer cells may exacerbate lentiviral vector losses during upstream processing

By incubating crude supernatant at 36.5 °C, the studies described in Section 6.2.1.1 established a rate of ProSavin® inactivation that is likely representative of that which occurs during standard upstream processing. However, as it is known that PS46.2 cells are autotransduced following induction of vector production (Stewart *et al.*, 2011), it was considered that these studies may underestimate the true extent of vector loss during upstream processing as, due to the preclusion of cells from supernatants during these studies, autotransduction did not occur. A study was thus devised to determine whether cell autotransduction is likely to lead to significant vector loss during upstream processing. In order to mimic the environment experienced by lentiviral vector particles during upstream processing as accurately as possible, it was desirable for vector losses to be measured over time in the presence of induced producer cells. In order to achieve this, it was necessary to use an EIAV lentiviral vector that expressed the *LacZ* marker gene, as this vector could be quantified in isolation from produced ProSavin® particles.

An experiment was devised where the proportion of LacZ vector remaining after 48 hr when incubated in the presence of cells that were or were not induced, was compared to the proportion remaining when incubated in fresh culture medium without cells (+/- induction reagents). The materials and methods for this study are described in full in Section 2.8.2, while the results (*LacZ* titre data) are presented in Figure 6.8. Surprisingly, in the presence of cells, around twice the amount of functional LacZ vector particles remained after 48 hr incubation, than when LacZ vector had been incubated in fresh medium alone (Figure 6.8). Subjecting this dataset to rigorous statistical testing would be inappropriate due to the inclusion of only a limited number of replicates (three per condition). However, it may be observed that the 95 % confidence intervals associated with LacZ titres, following 48 hr incubation, overlap in all instances except when comparing the 'medium + induction reagents' and 'cells + induction reagents' conditions (Figure 6.8). This indicates that the difference between these two conditions was likely statistically significant at the level $p < 0.05$, while no conclusion can be drawn regarding all other pairs of conditions. It was hypothesised that the presence of cells may have 'conditioned' the medium, altering its properties in a way that helped mitigate vector inactivation; therefore this was investigated in a second study (described below). Overall, this

first study suggests that vector losses due to the autotransduction of producer cells are likely to be negligible during upstream processing. However, firm conclusions are not possible due to the unexpected benefit conferred by the presence of cells in terms of vector stability (i.e. autotransduction may have led to vector losses, but any losses were outweighed by the stability benefits conferred by the presence of the cells).

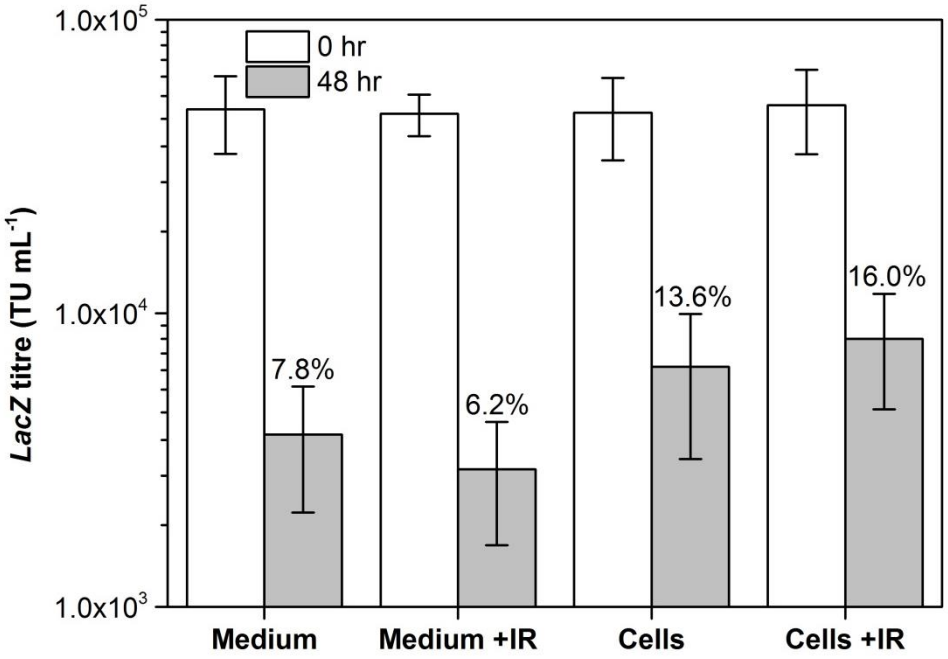


Figure 6.8 Reduction in *LacZ* biological titres over time when incubated in four different conditions (+/- cells; +/- induction reagents [IR]). The materials and methods for this study were described in Section 2.8.2. The culture medium in all instances was Freestyle™ 293 expression medium supplemented with 5 % (v/v) tet-free FCS. LacZ vector was added to all conditions 24 hr post-seeding (at the same time as induction reagents, where applicable). *LacZ* titres were quantified at this time (0 hr) and after a further 48 hr. Mean data is presented and error bars represent the 95 % confidence interval (n = 3). The percentage remaining after 48 hr is indicated for each condition.

In a second experiment, the proportion of LacZ vector remaining after 24 hr incubation when diluted 1 in 50 in standard media, which either had been pre-conditioned (i.e. spent media previously used for the culture of cells) or had not been pre-conditioned (i.e. fresh media) was compared. The materials and methods for this study are described in Section 2.8.2, while the results (*LacZ* titre data) are presented in Figure 6.9. It appeared that pre-conditioning the

culture medium conferred some small benefit in terms of lentiviral vector stability, as an average of 52.85 % LacZ vector remained after 24 hr incubation in pre-conditioned media, as compared to an average of 42.75 % in fresh media (combined averages of both suspension and adherent culture medium data). However, the 95 % confidence intervals associated with LacZ titres following 24 hr incubation overlapped in all instances (Figure 6.9), thus it was unlikely the observed differences between conditions were statistically significant. In the first experiment, the proportion of LacZ remaining was lower (6.2 - 16.0 %; Figure 6.8) than in the current experiment, and this was because a longer incubation period of 48 hr was used. It is therefore possible that clearer differences between the conditions examined in the current study (Figure 6.9) may have evolved had a 48 hr incubation period (such as that employed in the first experiment) been used. The osmolality, pH and electrolyte concentrations of cell culture media were measured, and this data revealed that both pre-conditioned media had a pH closer to neutral than the fresh media (Table 6.3). As it has been previously reported that lentiviral vectors decay at a faster rate when subjected to slightly alkaline conditions (Higashikawa and Chang, 2001), this offers a possible explanation for why the inclusion of cells in culture medium may moderate lentiviral vector inactivation. Lentiviral vector stability did not appear affected by the particular media type (i.e. suspension *versus* adherent) used (Figure 6.9), despite the differing properties of the two media formulations (Table 6.3). The insights obtained from this work have potential application with regard to the development of fed-batch or perfusion strategies for lentiviral vector production (the ratio of fresh:spent media should be considered) and when developing formulations for lentiviral vector storage.

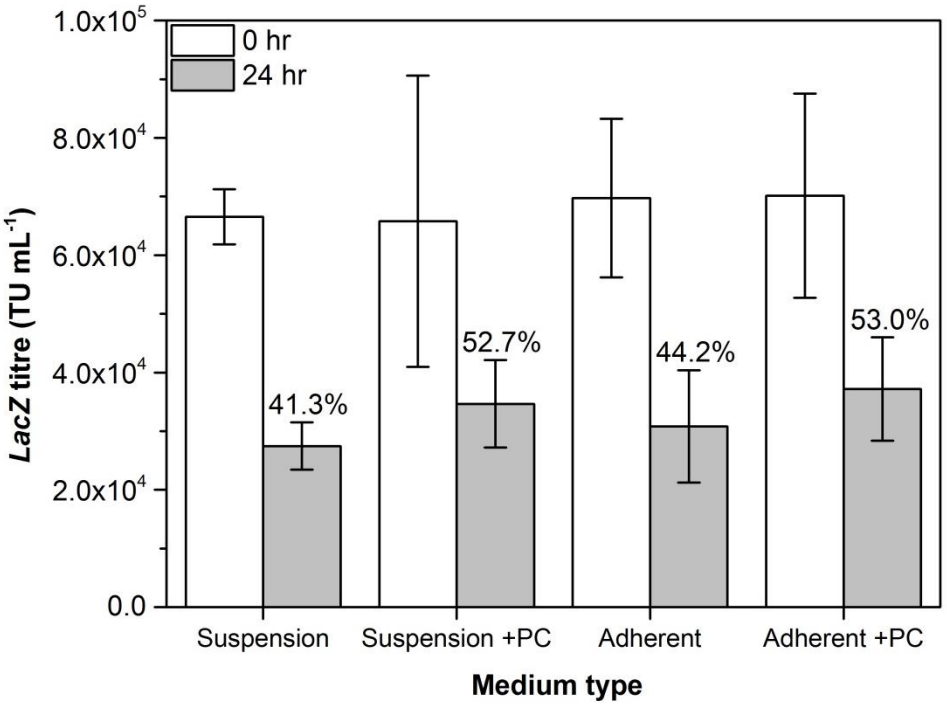


Figure 6.9 Reduction in *LacZ* biological titres over time when incubated in four different conditions (two different media; +/- pre-conditioning [PC]). The materials and methods for this study were described in Section 2.8.2. The suspension medium was Freestyle™ 293 expression medium supplemented with 5 % (v/v) tet-free FCS, while adherent medium was that typically used for HEK293T and D17 cell culture (see Section 2.1.4). 'Pre-conditioned' suspension medium had been previously used for the culture of non-induced suspension-adapted PS46.2; 'pre-conditioned' adherent medium had previously been used for the culture of HEK293T. LacZ vector was added to all conditions and *LacZ* titres were quantified at the start of the experiment (0 hr) and after 24 hr. Mean data is presented and error bars represent the 95 % confidence interval (n = 3). The percentage remaining after 24 hr is indicated for each condition.

Table 6.3 Osmolality, pH and electrolyte concentrations (Na^+ and Ca^{2+}) of cell culture media that had or had not previously been used for the cultivation of cells (i.e. pre-conditioned, PC). Media were as described in Figure 6.9. Note that samples were equilibrated in a 5 % CO_2 (36.5 °C) incubator before analysis, to obtain pH values most likely representative of those prevailing during the incubation of diluted lentiviral vector samples.

Medium type	Osmolality (osmol kg^{-1})	pH	Electrolyte concentrations (mmol L^{-1})	
			Na^+	Ca^{2+}
Suspension	0.275	7.4	106	0.29
Suspension +PC	0.263	7.2	111	0.29
Adherent	0.329	7.6	145	1.39
Adherent +PC	0.321	7.1	148	1.26

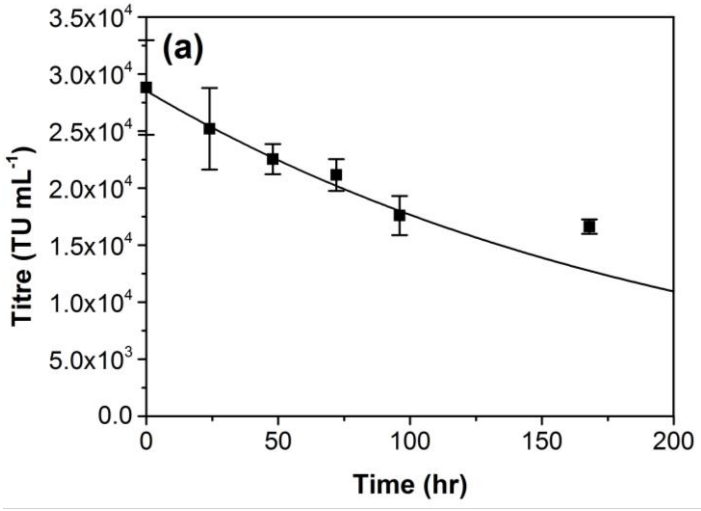
6.2.2 Evaluation of strategies to moderate or offset losses in functional ProSavin® particles due to their inactivation during upstream processing

Having characterised the stability of functional ProSavin®, vector RNA and vector RT under typical production conditions (Section 6.2.1.1), the next objective was to trial approaches for mitigating ProSavin® inactivation that could be applied during upstream processing. A review of related literature revealed three strategies of potential relevance for the ProSavin® process. These were based on (i) the storage of generated vector at 4 °C (of relevance for the development of perfusion-based processes), (ii) a reduction in the temperature at which ProSavin® is produced (to 31.5 °C), and (iii) adjustment of the osmotic properties of the culture medium using glucose, fructose and/or sorbitol. The background to each approach, and the results of its application to the ProSavin® process, are outlined below.

6.2.2.1 Storage at 4 °C

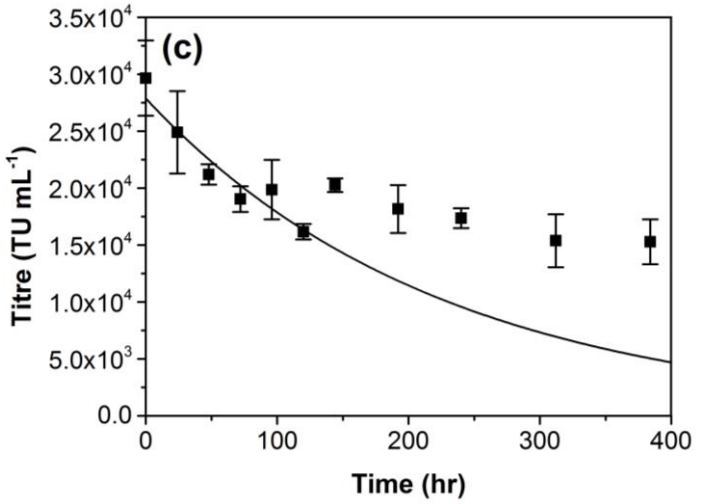
Although HIV-1 derived lentiviral vectors pseudotyped with VSV-G have been reported to display a longer half-life when stored at 4 °C as compared to 37 °C (Higashikawa and Chang, 2001), the stability of EIAV-derived lentiviral vectors at lowered temperatures was not known. Consequently, to inform the development of enhanced manufacturing procedures for EIAV-derived lentiviral vectors, two studies were conducted to assess whether storage of ProSavin® at 4 °C could moderate the rapid inactivation observed at 36.5 °C. Initially, supernatants harvested from standard shake flask cultures of PS46.2 were aliquoted into vials and refrigerated for various time periods of up to 168 hr (Section 2.9.1). Temperatures were monitored throughout the study using a TempTale®4 (Sensitech®) temperature monitor (readings were taken at intervals of 30 min), and the average temperature (± 1 SD) was observed to be 4.1 ± 0.5 °C, with a range of 3.3 to 5.3 °C. ProSavin® titres were subsequently quantified and this data is presented in Figure 6.10 (a). Titres remained high throughout the study, with 58 % remaining after 168 hr (7 days). Linear regression analysis of natural log transformed titre data revealed that functional ProSavin® decayed with a half-life of around 144.4 hr during the first 96 hr (Figure 6.10 [b]). Note that, in order to achieve the best model fit, the 168 hr data points were excluded from the linear regression analysis. The difference between the 96 hr and 168 hr data points was negligible (1.76×10^4 versus 1.66×10^4 TU mL^{-1},

respectively), suggesting that the decay rate considerably slowed or halted during this period. To verify this observation, a second experiment was conducted in which supernatants harvested from standard shake flask cultures of PS46.2 were aliquoted into vials and refrigerated for various time periods of up to 384 hr (16 days; Section 2.9.1). Again, temperatures were monitored every 30 min for the duration of the study using a TempTale®4 temperature monitor, and the average temperature (± 1 SD) was found to be 4.2 ± 0.5 °C, with a range of 2.9 to 5.7 °C. ProSavin® titre data is presented in Figure 6.10 (c). The results support the earlier findings that, although titres declined steadily during the initial ~ 96 hr of cold storage, the decay profile subsequently levelled out, and after 384 hr (16 days) 52 % of the initial titre remained. Variability in the data points made it difficult to pinpoint exactly when this shift in decay rate occurred (Figure 6.10 [c]), thus linear regression analysis was performed using natural log transformed titres obtained between 0 and 96 hr, in agreement with the earlier study (Figure 6.10 [a] and [b]). From this analysis, the half-life of ProSavin® was calculated to be 150.7 hr over this time period (Figure 6.10 [d]), which is reasonable agreement with the half-life calculated during the first study (144.4 hr, Figure 6.10 [b]). The stability of vector RNA at 4 °C over a period of 384 hr was also examined (Figure 6.11). No discernable reduction in RNA copy number was observed, which supports the earlier conclusion based on the 36.5 °C stability data that RNA degradation is unlikely to be the primary mechanism for ProSavin® inactivation. The data indicate that refrigeration of harvested ProSavin®-containing supernatants can prevent vector RNA degradation for at least up to 16 days.



(b)

Intercept (*a*)	Slope (*b*)	R^2	*p* value	$t_{½}$ (hr)
10.26 ± 0.09	-0.0048 ± 0.0016	0.762	< 0.0001	144.4 (108.3, 216.6)



(d)

Intercept (*a*)	Slope (*b*)	R^2	*p* value	$t_{½}$ (hr)
10.24 ± 0.10	-0.0046 ± 0.0016	0.644	< 0.0001	150.7 (111.8, 231.0)

Figure 6.10 Functional ProSavin® stability profiles during the storage of crude supernatant at 4 °C. Titres were quantified using the rapid ProSavin® titre assay (Section 2.11.2.1). Results from two separate studies are shown, in which ProSavin® was stored at 4 °C for a period of 0 - 168 hr (titre data [a] and linear regression results [b]) or 0 - 384 hr (titre data [c] and linear regression results [d]). Experimental procedures were as described in Section 2.9.1. In plots (a) and (c) mean data is presented and error bars represent ± 1 SD (n = 3 [a] or n = 4 [c]). Exponential trend lines were constructed according to the method described in Figure 6.1, using results obtained from linear regression analysis of natural log transformed data (linear fitting was conducted using data obtained between 0 and 96 hr only). Slope and intercept values include a 95 % confidence interval. The half-life of functional ProSavin® (with upper and lower 95 % confidence limits) was calculated using Equation 6.1 as described in Section 6.2.1.1.

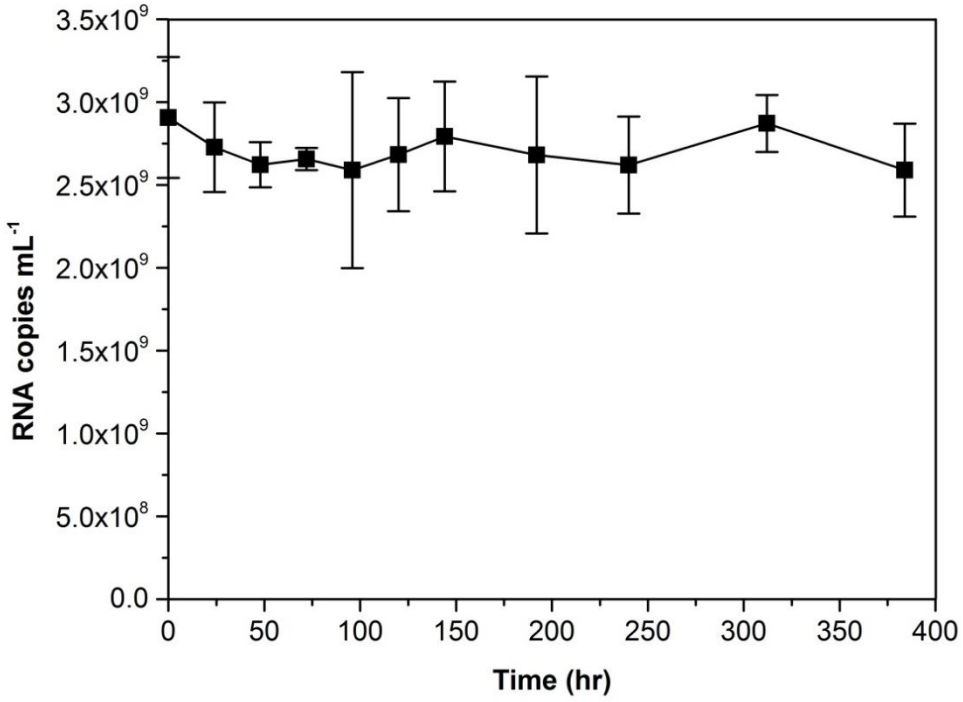


Figure 6.11 Vector RNA stability profile during the storage of crude supernatant at 4 °C. ProSavin® was stored at 4 °C for a period of 0 - 384 hr; experimental procedures were as described in Section 2.9.1. Mean data is presented and error bars represent ± 1 SD (n = 4).

6.2.2.2 Production at 31.5 °C

Various studies using gamma-retroviral vector production systems have reported improved titres following a reduction in culture temperature from 37 °C to 32 °C due, at least in part, to the increased stability of gamma-retroviral vectors at the lower temperature (Kotani *et al.*, 1994; Lee *et al.*, 1996; Kaptein *et al.*, 1997; McTaggart and Al-Rubeai, 2000). However, this strategy has not been applied to lentiviral vector production. Consequently, a study was conducted to evaluate whether lowering the temperature by 5 °C (to 31.5 °C) from typical production temperatures (36.5 °C) could improve ProSavin® titres. ProSavin® was generated using shake flask cultures of PS46.2 incubated either at 31.5 or 36.5 °C. Standard operating procedures were broadly followed, except that flasks were seeded using a slightly higher than normal cell density (1.6 x 10^6 viable cells mL^{-1}) and were induced after 2 hr to ensure similar numbers of cells were induced in both conditions. If inductions were performed after 24 hr, potential differences in cell growth rate at the two temperatures could lead to different numbers of cells

being induced, which would distort the data. Harvests were performed at 24, 44 and 67 hr post-induction. The experimental procedure for this study is outlined in full in Section 2.9.2.

Cell growth was monitored throughout the culture period and this data is presented in Figure 6.12. Little cell growth was observed in shake flasks incubated at either temperature, and peak viable cell concentrations observed 26 hr post-seeding were 2.0 x 10^6 and 1.8 x 10^6 viable cells mL^{-1} for cultures incubated at 31.5 and 36.5 °C, respectively. Viable cell concentrations, as well as cell viabilities, remained highest in the cultures incubated at 31.5 °C (Figure 6.12). At the end of the culture period (69 hr post-seeding), 1.3 x 10^6 viable cells mL^{-1} remained in the cultures incubated at 31.5 °C, as compared to 4.3 x 10^5 viable cells mL^{-1} in those incubated at 36.5 °C, while viabilities were 75 % *versus* 34 %, respectively. Although reduction of the culture temperature to 31.5 °C enabled greater cell survival, titres were compromised (Figure 6.13). At both temperatures, the highest titres were obtained at 24 hr post-induction. However, maximum titre values attained from the 31.5 °C cultures were just 5.6 x 10^3 TU mL^{-1}, around five-fold lower than those attained from the 36.5 °C cultures (2.7 x 10^4 TU mL^{-1}). Thus, even if ProSavin® generated using a production temperature of 31.5 °C did decay at a relatively slower rate than ProSavin® produced at 36.5 °C (which was not tested), any potential benefit was far outweighed by the reduced productivity of these cultures, indicating that lowered temperature cultivation of PS46.2 is unlikely to prove an effective strategy for enhancing titres upstream.

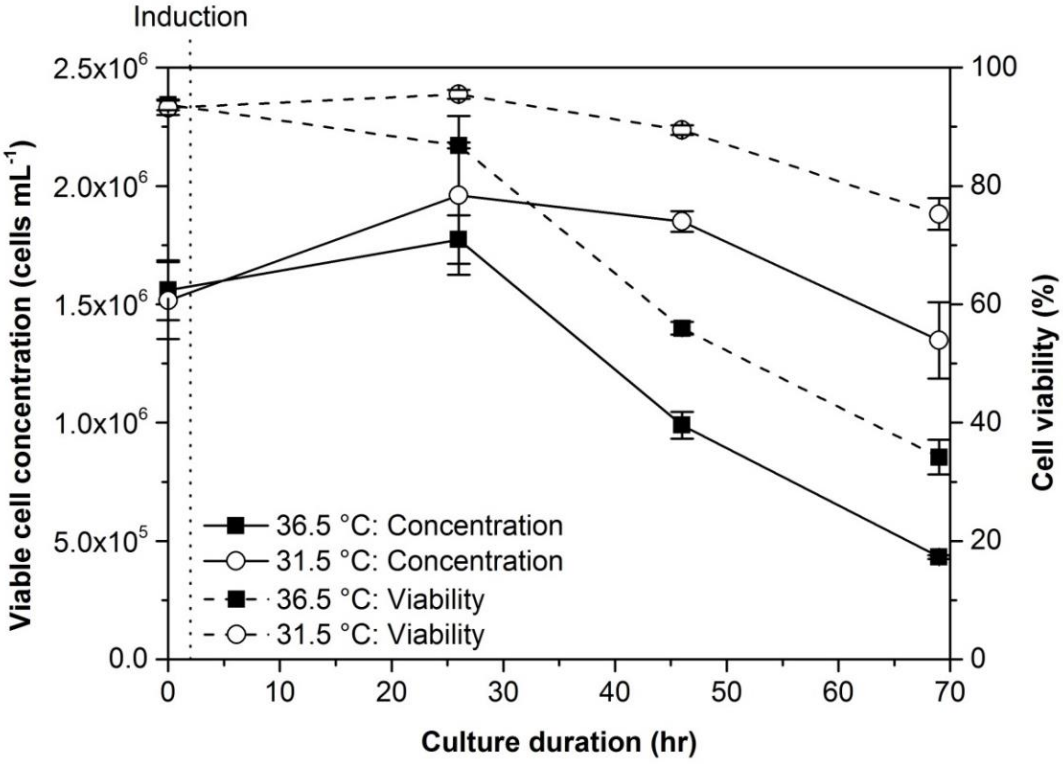


Figure 6.12 Viable cell concentration and viability of PS46.2 cells over time when cultured using either standard (36.5 °C) or reduced (31.5 °C) temperatures. Shake flask cultures were seeded at a density of 1.6 x 10^4 viable cells mL^{-1}, and were induced 2 hr later as indicated by the dotted line. The materials and methods for this study are described in full in Section 2.9.2. Mean data is presented and error bars represent ± 1 SD (n = 4).

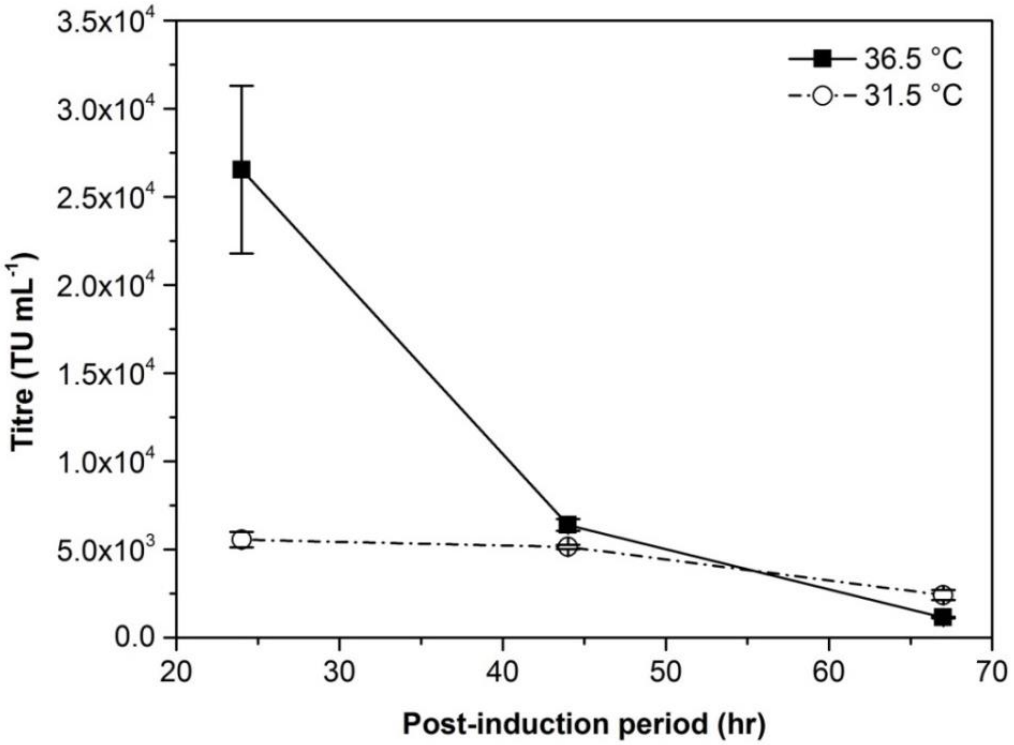


Figure 6.13 ProSavin® titres (quantified according to Section 2.11.2.1) generated using PS46.2 cells cultured using either standard (36.5 °C) or reduced (31.5 °C) temperatures. Experimental procedures were as noted for Figure 6.12. Mean data is presented and error bars represent ± 1 SD (n = 4).

6.2.2.3 Adjustment of medium osmolality

Recent studies have demonstrated that supplementation of the culture medium with different sugar types alters its osmotic properties and can improve retroviral vector yields (Coroadinha *et al.*, 2006a; 2006b; 2006c), although this approach has not been applied to lentiviral vector production. The purpose of this study was to trial whether adjustment of the sugar content and thus osmolality of the culture medium could confer any benefit for the ProSavin® process. In order to establish the background and rationale for this study, a brief overview of the relevant literature (Coroadinha *et al.*, 2006a; 2006b; 2006c) is first provided.

All studies described by Coroadinha *et al.* (2006a), (2006b) and (2006c) were based on adherent culture of a human HT1080-derived retroviral (Moloney murine leukaemia virus) vector producer cell line, and employed a standard medium comprised of DMEM supplemented with 10 % (v/v) FCS, 6 mM glutamine and 25 mM glucose, which had an osmolality of around 0.325

- 0.335 osmol kg^{-1}. Coroadinha *et al.* (2006a) reported that supplementation of this standard medium with 140 mM fructose enhanced titres four-fold, while Coroadinha *et al.* (2006b) demonstrated a similar improvement when using 140 mM glucose or 140 mM fructose in place of the 25 mM glucose included in standard medium, and also noted that the medium osmolality was increased to 0.464 osmol kg^{-1} as a result of these modifications. Coroadinha *et al.* (2006c) reported that raising the osmolality of the standard medium to between 0.410 and 0.450 osmol kg^{-1} by the addition of sorbitol (the concentration of which was not reported) yielded an improvement in titres of up to two-fold. The titre improvements described in these papers were attributed to the combined effect of increased cellular productivity and improved vector stability (Coroadinha *et al.*, 2006a; 2006b; 2006c). A decrease in the cholesterol to phospholipid ratio of vector membranes was found to be responsible for the enhanced vector stability observed at increased osmolalities (Coroadinha *et al.*, 2006c).

In the current work, the effect of increased osmolality attained by supplementation of the medium with glucose, fructose and/or sorbitol on ProSavin® titres was evaluated using microwell suspension cultures of PS46.2. In order to evaluate a broad range of conditions, and thus maximise the likelihood of capturing any that could confer benefit, a full factorial screening experiment was designed, where the concentration of each sugar type was assessed, either singularly or in combination, over the range 0 - 100 mM as indicated in Table 6.4. A number of factors defined the concentration range evaluated in this work. Firstly, the standard culture medium used in this work (FreeStyle 293™ expression medium supplemented with 5 % [v/v] tet-free FCS) already contained glucose at an estimated concentration of around 23 - 25 mM (based on previous analysis of two different medium batches using the YSI instrument [methods Section 2.11.1.4]). Note that the glucose content of FreeStyle 293™ expression medium is not specified by the manufacturer, presumably as this information is proprietary. Further supplementation using 100 mM glucose, fructose *or* sorbitol (as in runs 2, 3 and 5; Table 6.4) therefore took the final concentration of sugars in the culture medium to around 123 - 125 mM, which was within the range reported by Coroadinha *et al.* (2006b) to be beneficial for retroviral vector production. For all other runs supplemented with a combination of sugars (runs 4 and 7 - 12; Table 6.4), the final concentration of sugars was higher than that previously investigated by Coroadinha *et al.* (2006a) or (2006b). However, as Coroadinha *et al.* (2006c) reported that a

medium osmolality of 0.410 and 0.450 osmol kg^{-1} adjusted by the addition of an unknown quantity of sorbitol was optimal for retroviral vector production, these conditions were included to allow a greater osmotic range to be evaluated (runs 8-12 yielded osmotic conditions close to or within this range; Table 6.4). The low concentration setting evaluated in this work (0 mM; i.e. no supplementation) provided a baseline against which the effect of sugar additions could be assessed (run 1).

Cells were cultivated in the various media (Table 6.4) according to standard shake flask procedures for an acclimatisation period of three days. The concentration and viability of PS46.2 cells following this period is detailed in Appendix C. To control for variability in the growth rate of PS46.2 when cultured in the different media conditions, all microwells were seeded using the same density of viable cells (2×10^6 viable cells mL^{-1}) and were induced after 2 hr for evaluation of ProSavin® production. ProSavin® harvests were performed at 23, 44 and 65 hr post-induction. The materials and methods used for this study are outlined in full in Section 2.9.3.

Table 6.4 Experimental design matrix for investigation into the effect of medium osmolality (adjusted by the addition of fructose, glucose and/or sorbitol) on cell growth and lentiviral vector production. The concentration of each sugar type was varied over two levels as indicated, and the resulting osmolality of each medium is given (n = 1). Four centre point experiments were also included for estimation of pure error. Each medium was prepared as outlined in Section 2.9.3. REF denotes the standard medium without supplementation, or partial dilution with water (REF is therefore distinct from run 1, which contained an equivalent proportion of tissue culture grade water [9.3 % (v/v)] to that included in runs 2 - 12; see Section 2.9.3 for more details).

Run	Concentration of osmotic agent (mM)			Medium osmolality (osmol kg^{-1})
	Fructose	Glucose	Sorbitol	
1	-	-	-	0.255
2	100	-	-	0.354
3	-	100	-	0.361
4	100	100	-	0.473
5	-	-	100	0.346
6	100	-	100	0.471
7	-	100	100	0.470
8	100	100	100	0.456
9 - 12	50	50	50	0.412
REF	-	-	-	0.275

Cell growth data, collected at the time of vector harvests (23, 44 and 65 hr post-induction), is presented in Figure 6.14. Cell numbers initially increased in the cultures that were either not supplemented (run 1 and REF) or had been supplemented with just one sugar (runs 2, 3 and 5), reaching a concentration of 3.1 - 4.4 x 10^6 viable cells mL^{-1} by 23 hr post-induction. In all other cultures, by 23 hr post-induction cell numbers had declined from their starting concentration of 2.0 x 10^6 viable cells mL^{-1} to 1.1 - 1.7 x 10^6 viable cells mL^{-1} and continued to decline thereafter, with the exception of run 8, where cell numbers subsequently increased, reaching 2.4 x 10^6 viable cells mL^{-1} by 65 hr post-induction. These findings are broadly consistent with those of Coroadinha *et al.* (2006a), (2006b) and (2006c), who reported reduced growth of HT1080-derived retroviral vector producer cells when the standard medium (containing 25 mM glucose) was further supplemented with 140 mM fructose, 115 mM glucose, or sorbitol to an osmolality of 0.410 - 0.500 osmol kg^{-1}.

Cell viabilities followed a similar trend (Figure 6.14 [b]). Viabilities were 91.2 - 95.0 % at 23 hr post-induction in cultures supplemented with 0 mM (run 1 and REF) or 100 mM (runs 2, 3 and 5) sugar, and 31.4 - 78.0 % in all other runs. Viabilities declined with subsequent harvests except in the case of run 8, where they increased (from 31.4 to 43.0 %). Overall, the data suggest that PS46.2 were able to cope well with the addition of one sugar type to the culture medium (100 mM concentration; osmolality 0.346 - 0.361 osmol kg^{-1}), however were severely compromised when more than one sugar type was added to a final concentration of 150 - 200 mM (osmolality 0.412 - 0.473 osmol kg^{-1}). Interestingly, PS46.2 appears to have been able to adapt to the most extreme media condition evaluated - combined sugar supplementation to a final concentration of 300 mM (osmolality 0.456 osmol kg^{-1}).

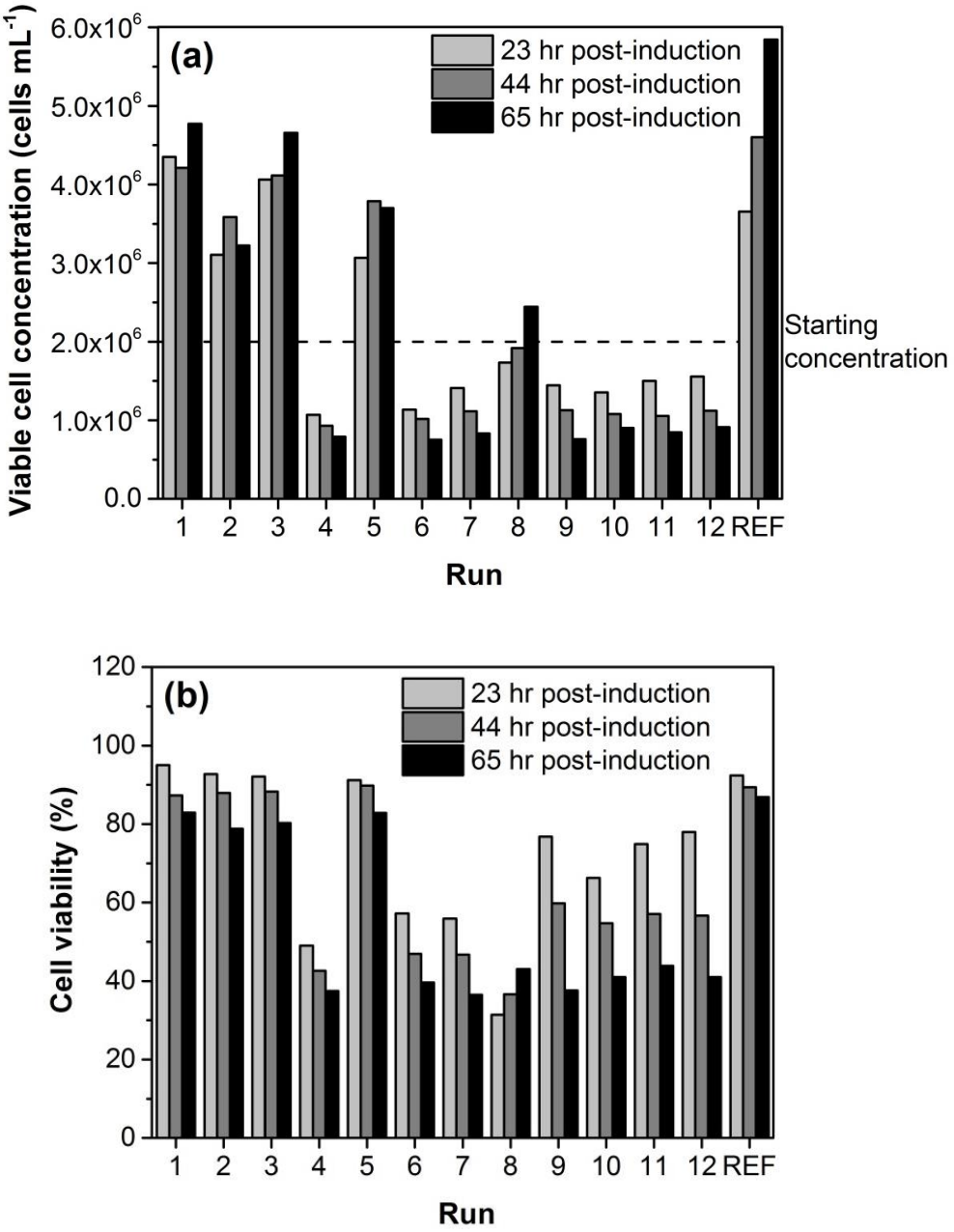


Figure 6.14 Viable cell concentration (a) and cell viability (b) of induced microwell cultures of PS46.2 cultivated in medium supplemented with different sugar types. Measurements were performed at 23, 44 and 65 hr post-induction. Cells were seeded at a density of 2 x 10^6 viable cells mL^{-1}, as indicated by the dashed line (a), and were induced 2 hr later. Experimental conditions are indicated by the run number (see Table 6.4). Note that the cells had been cultivated in shake flasks in the various media (Table 6.4) for an acclimatisation period of three days prior to initiation of this experiment. The full materials and methods for this study are described in full in Section 2.9.3. Data from individual microwells is presented.

ProSavin® titres at 23, 44 and 65 hr post-induction are presented in Figure 6.15. The highest titres, 5.4 x 10^4 TU mL^{-1} and 4.4 x 10^4 TU mL^{-1}, were observed at 44 hr post-induction in the cultures that had not been supplemented with sugars (REF and run 1, respectively). Moderate titres (maximum: 2.1 - 2.9 x 10^4 TU mL^{-1}) were attained from the cultures that had been supplemented with 100 mM fructose, glucose or sorbitol (runs 2, 3 and 5), while low titres (maximum: 3.2 - 5.2 x 10^3 TU mL^{-1}) were obtained from cultures where all three sugar types had been added to a final concentration of 150 mM (runs 9 - 12). Cultures supplemented with two or three sugar types to a final concentration of 200 - 300 mM (runs 4, 6, 7 and 8) generated little or no functional ProSavin®. The proportion of cells transduced by these samples was 0.00 - 0.04 %, the same proportion as was transduced in the negative control populations (n = 6), indicating that any positive values may be attributed to assay noise due to background fluorescence. Titre data has also been plotted as a function of culture osmolality (Figure 6.16). Overall, it is clear that increasing culture osmolalities, whether engineered by the addition of fructose, glucose and/or sorbitol, are progressively detrimental for ProSavin® titres.

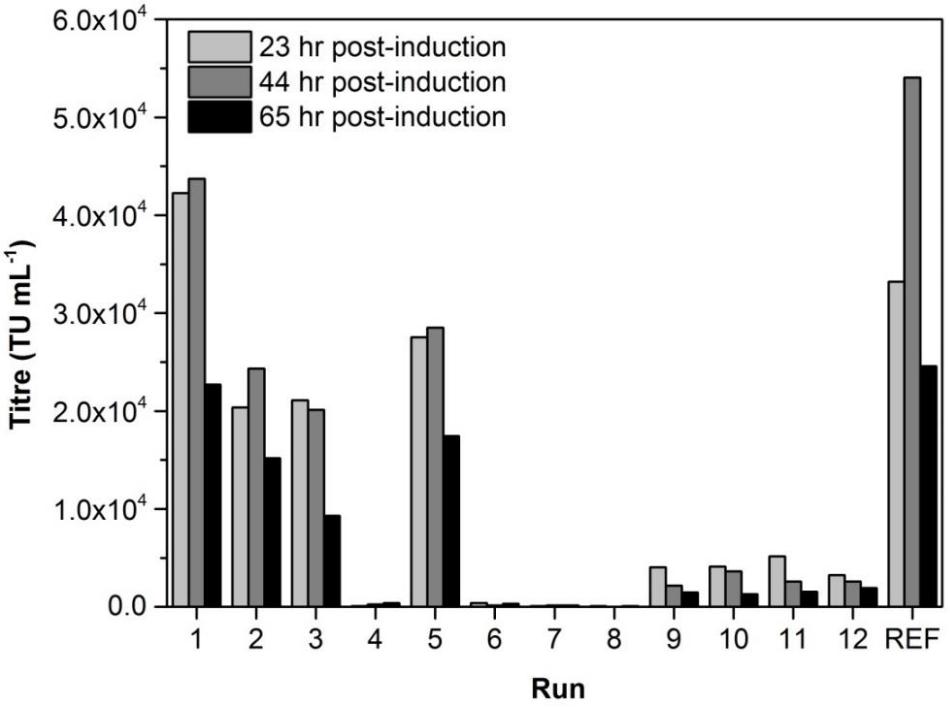


Figure 6.15 ProSavin® titres (quantified according to Section 2.11.2.1) at 23, 44 and 65 hr post-induction in microwell cultures of PS46.2 supplemented with different sugar types. Experimental conditions are indicated by the run number (see Table 6.4). Procedures were as noted for Figure 6.14. Data from individual microwells is presented.

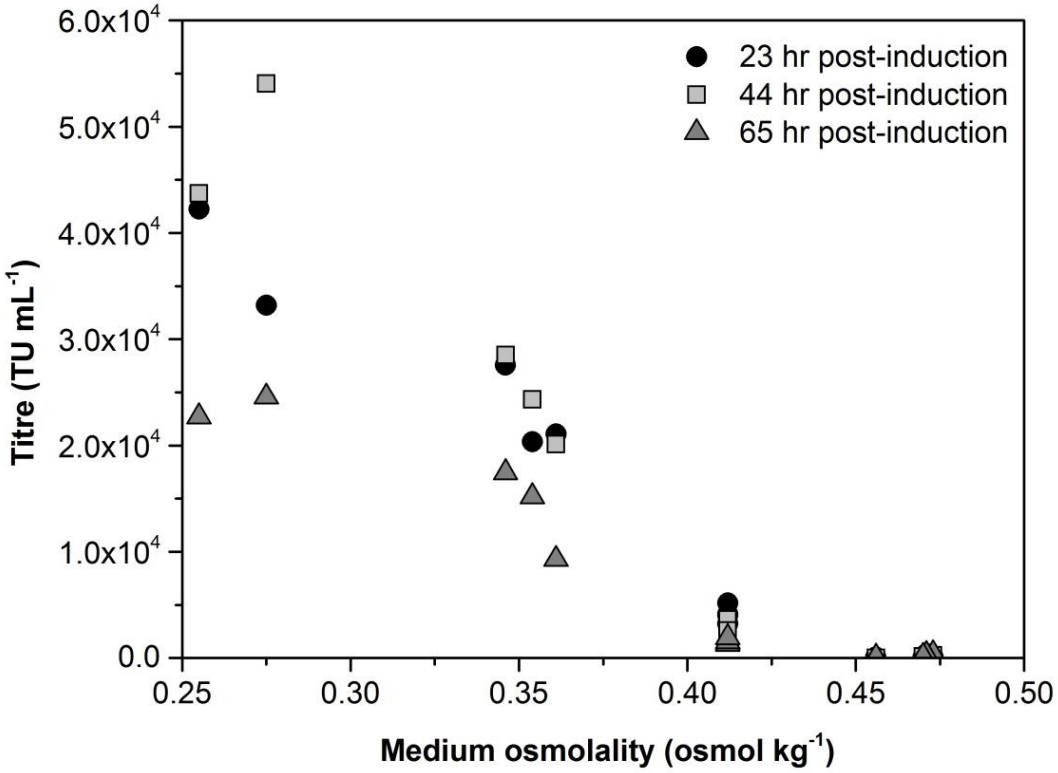


Figure 6.16 Scatter plot illustrating ProSavin® titres (quantified according to Section 2.11.2.1) in relation to medium osmolality. Titre data is the same as that presented in Figure 6.15, and was obtained following the culture of PS46.2 in microwells supplemented with different sugar types. Materials and methods were as noted for Figure 6.14. Data from individual microwells is presented.

Lastly, to verify that altering the sugar content of the culture medium had not impacted on the transduction stage of the ProSavin® titre assay, ProSavin®-containing supernatants that had been harvested from standard shake flask cultures (operated in parallel to microwells) were adjusted to contain sugars at the concentrations specified in Table 6.4. ProSavin® titres were then quantified as normal using the flow cytometry method described in Section 2.11.2.1. As illustrated in Figure 6.17, adjustment of the sugar content of the supernatants prior to performing cell transductions had no effect on ProSavin® titres (the variability between conditions was within the typical range observed for identical replicates, e.g. see Appendix B, Figure B.1).

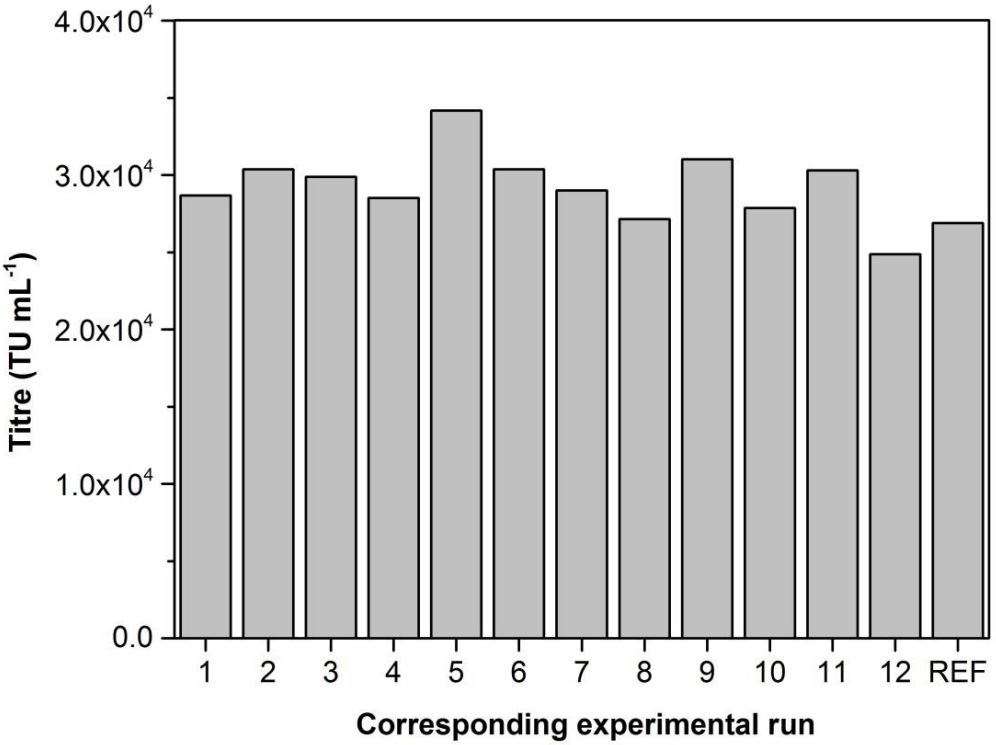


Figure 6.17 The influence of sugar supplementation on cell transductions. ProSavin®-containing supernatants that had been harvested from standard shake flask cultures (Section 2.4.1.3) were supplemented to achieve resulting concentrations of fructose, glucose and sorbitol as stated in Table 6.4, following which titres were quantified according to standard protocol as described in Section 2.11.2.1 (n = 1). The methods for this study are described in Section 2.9.3.

6.2.3 Broader evaluation of EIAV lentiviral vector stability at 36.5 °C

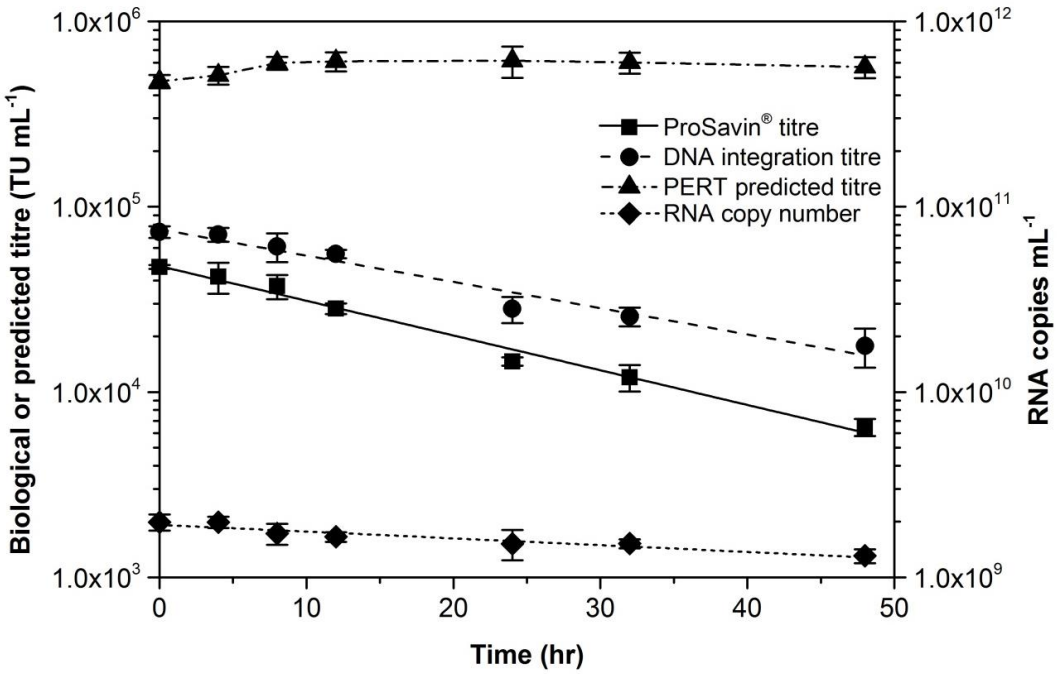
6.2.3.1 ProSavin® generated using adherent PS46.2 or transient transfection techniques

Earlier, the half-life of functional ProSavin®, vector RNA and reverse transcriptase (RT) in crude supernatants incubated at 36.5 °C was established (Section 6.2.1.1). Functional ProSavin® predominantly decayed with a half-life of 10.1 hr (Figure 6.5), while vector RNA was more robust and exhibited a half-life of 23.3 hr (Figure 6.6). PERT predicted titres (based on a measurement of vector RT activity) did not reduce during the first 48 hr incubation, after which they declined slowly, with a half-life of around 80.6 hr (Figure 6.7). The ProSavin® utilised in these experiments had been generated using suspension cultures of PS46.2. To discover whether the stability characteristics of ProSavin® depended on the particular production method employed, the decay kinetics of ProSavin® that had been generated using adherent cultures of

PS46.2 (Section 2.10.2) or by transient transfection of adherent HEK293T (Section 2.10.3) were here also examined. Crude supernatants harvested from PS46.2 or HEK293T cultures were incubated at 36.5 °C for varying lengths of time up to 48 or 24 hr, respectively. The time points were chosen such that the depletion of functional vector to low levels, but still within the quantifiable range of the assays, might be observed (see Section 2.10.1 for a full description of the methods employed). Functional titres were quantified using the ProSavin® titre assay (Section 2.11.2.1) and, to facilitate later comparison with the results presented in Section 6.2.3.2, the DNA integration assay (Section 2.11.2.3). PERT predicted titres and RNA copy numbers were also quantified.

The half-life of functional ProSavin® derived from adherent cultures of PS46.2 was calculated to be 16.1 hr based on results obtained using the ProSavin® titre assay and 21.2 hr based on results obtained using the DNA integration assay (Figure 6.18). Some discrepancy between the two methods was expected as, following the transduction of target cells, each assay measures a different parameter - the DNA integration assay measures integrated proviral DNA using qPCR, while the ProSavin® titre assay measures transgene expression using flow cytometry. Starting DNA integration titres were 7.3 x 10^4 TU mL^{-1}, 55 % higher than starting ProSavin® titres (4.7 x 10^4 TU mL^{-1}). The difference in half-life subsequently observed could indicate that ProSavin® particles lose their capacity to transfer working copies of the transgene, specifically the TH enzyme component, earlier than they become unable to transfer complete copies of the EIAV Ψ which represents the region amplified and quantified using qPCR. Vector RNA decayed at a slower rate than functional particles (half-life 82.3 hr; Figure 6.18), which supports the notion that RNA degradation is unlikely to be the primary mechanism for ProSavin® inactivation (Section 6.2.1.1). The stability of functional ProSavin® and vector RNA derived from adherent cultures of PS46.2 was superior to that derived from suspension cultures of PS46.2 (Figures 6.5 and 6.6). PERT predicted titres did not decline over the time period examined here (48 hr), which was consistent with earlier data (Figure 6.7), and suggests that the RT enzyme associated with ProSavin® particles is relatively robust.

(a)



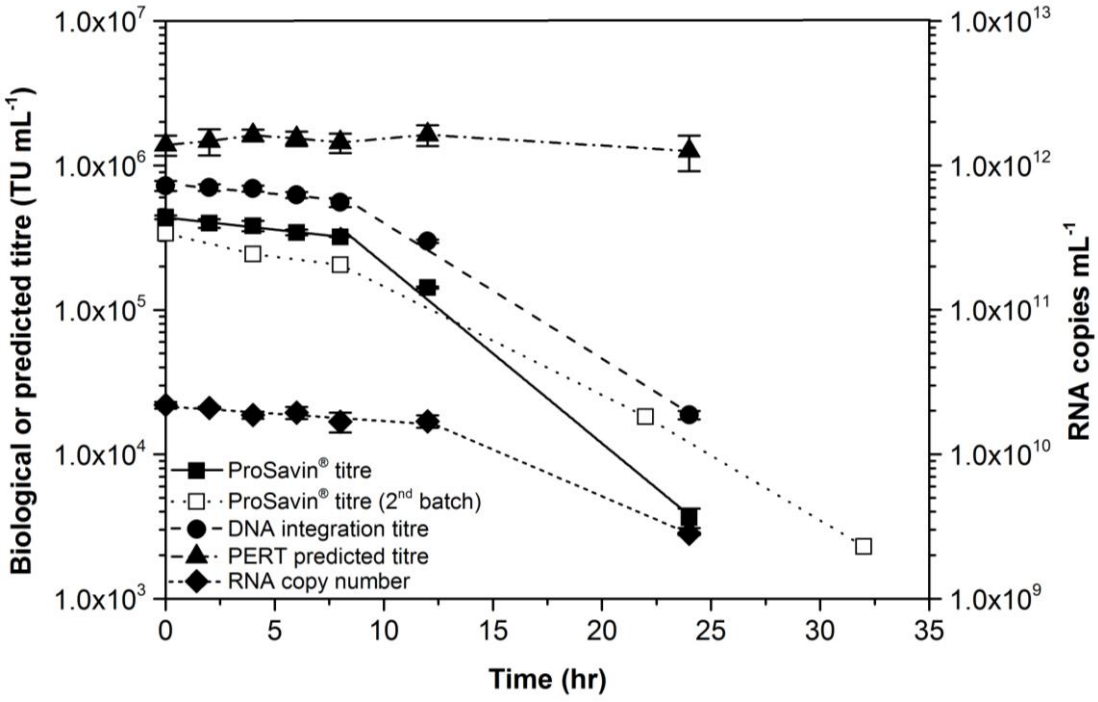
(b)

Measured response	Intercept (*a*)	Slope (*b*)	R^2	*p* value	$t_{½}$ (hr)
ProSavin® titre	10.78 ± 0.09	-0.0431 ± 0.0037	0.969	< 0.0001	16.1 (14.9, 17.6)
DNA integration titre	11.23 ± 0.11	-0.0326 ± 0.0046	0.920	< 0.0001	21.2 (18.6, 24.8)
RNA copy number	21.38 ± 0.07	-0.0084 ± 0.0023	0.647	< 0.0001	82.3 (60.7, 127.7)

Figure 6.18 Stability of functional ProSavin®, vector RT and RNA during the incubation of crude supernatant (generated from adherent cultures of PS46.2) at 36.5 °C. Functional (biological) titres were quantified using two methods: the ProSavin® titre assay (based on flow cytometry; Section 2.11.2.1) and the DNA integration assay (Section 2.11.2.3). In addition, titres were predicted based on a measurement of vector RT activity (PERT predicted titre; Section 2.11.3.2) and vector RNA copy numbers were also quantified (Section 2.11.3.1). The experimental procedure was as described in Section 2.10. Mean data is presented in plot (a) and error bars represent ± 1 SD (n = 3). Exponential trend line construction was completed using the method detailed in Figure 6.1, using the results of linear regression analysis presented in table (b). Note that PERT predicted titres were not analysed by linear regression, thus a trend line has not been fitted to this data. Values were transformed by taking the natural logarithm prior to linear regression analysis. Slope and intercept values include a 95 % confidence interval. The half-life of functional ProSavin® and vector RNA (with upper and lower 95 % confidence limits) was calculated using Equation 6.1 as described in Section 6.2.1.1.

Functional ProSavin® derived from adherent cultures of HEK293T, which had been transiently transfected using three plasmids, displayed a unique stability profile that was characterised by two relatively discrete inactivation phases (Figure 6.19). ProSavin® titres declined with a half-life of 18.0 hr during the initial 8 hr incubation, after which titres plummeted, displaying a half-life of 2.4 hr until the end of the incubation period (24 hr). DNA integration titres exhibited a similar pattern: initially (between 0 and 8 hr) titres declined with a half-life of 21.5 hr, after which they fell rapidly, with a half-life of 3.2 hr. The sudden rapid decline in functional ProSavin® observed after 8 hr incubation was surprising, and to verify this result a second batch of material was produced and tested (n = 1). A similar biphasic stability profile was observed (Figure 6.19), indicating that these results are likely representative of the decay kinetics of ProSavin® derived from transiently transfected HEK293T cultures. Interestingly, titres generated using this system were high as starting titre values were approximately ten-fold greater than those obtained from adherent cultures of PS46.2. This suggests that during a standard production run either vector production rates are high enough to compensate for vector losses due to inactivation, or harvests are completed before the second (accelerated) inactivation phase is fully underway (harvests are typically performed 21 - 23 hr post-medium exchange; see Stewart *et al.* (2009), (2011), and Section 2.10.3). Vector RNA exhibited a similar stability profile, decaying at a rate only marginally slower than functional particles (Figure 6.19). RNA copy numbers declined slowly, with a half-life of 29.6 hr during the initial 12 hr incubation, after which they declined at an accelerated rate of approximately 4.6 hr. The shift to a more rapid decay rate occurred 4 hr later than was observed for functional ProSavin®, lending weight to the conclusion that RNA degradation is unlikely to be a primary cause of ProSavin® inactivation. PERT predicted titres remained constant throughout the incubation period (24 hr), consistent with earlier observations (Figures 6.7 and 6.18).

(a)



(b)

Measured response	Intercept (*a*)	Slope (*b*)	R^2	*p* value	$t_{½}$ (hr)
ProSavin® titre					
1st phase (0-8 hr)	12.99 ± 0.05	-0.0385 ± 0.0098	0.847	< 0.0001	18.0 (14.4, 24.2)
2nd phase (8-24 hr)	15.11 ± 0.34	-0.2864 ± 0.0209	0.993	< 0.0001	2.4 (2.3, 2.6)
DNA integration titre					
1st phase (0-8 hr)	13.52 ± 0.06	-0.0323 ± 0.0130	0.688	0.0001	21.5 (15.3, 36.0)
2nd phase (8-24 hr)	15.06 ± 0.24	-0.2163 ± 0.0150	0.994	0.0006	3.2 (3.0, 3.4)
RNA copy number					
1st phase (0-12 hr)	23.79 ± 0.08	-0.0234 ± 0.0116	0.532	< 0.0001	29.6 (19.8, 58.9)
2nd phase (12-24 hr)	25.34 ± 0.26	-0.1491 ± 0.0136	0.996	< 0.0001	4.6 (4.3, 5.1)

Figure 6.19 Stability of functional ProSavin®, vector RT and RNA during the incubation of crude supernatant (generated by transient transfection using adherent cultures of HEK293T) at 36.5 °C. Biological (ProSavin® and DNA integration) titres, PERT predicted titres and RNA copy numbers were quantified as noted for Figure 6.18 (full experimental procedures were as outlined in Section 2.10). Mean data is presented in plot (a) and error bars represent ± 1 SD (n = 3), except in the case of the ProSavin® titre (2nd batch) data, where n = 1. Exponential trend line construction was completed (except in the case of the ProSavin® titre [2nd batch] and PERT data) according to the method detailed in Figure 6.1, using the results of linear regression analysis presented in table (b). Values were transformed by taking the natural logarithm prior to linear regression analysis. Slope and intercept values include a 95 % confidence interval. The half-life of functional ProSavin® and vector RNA (with upper and lower 95 % confidence limits) was calculated using Equation 6.1 as described in Section 6.2.1.1.

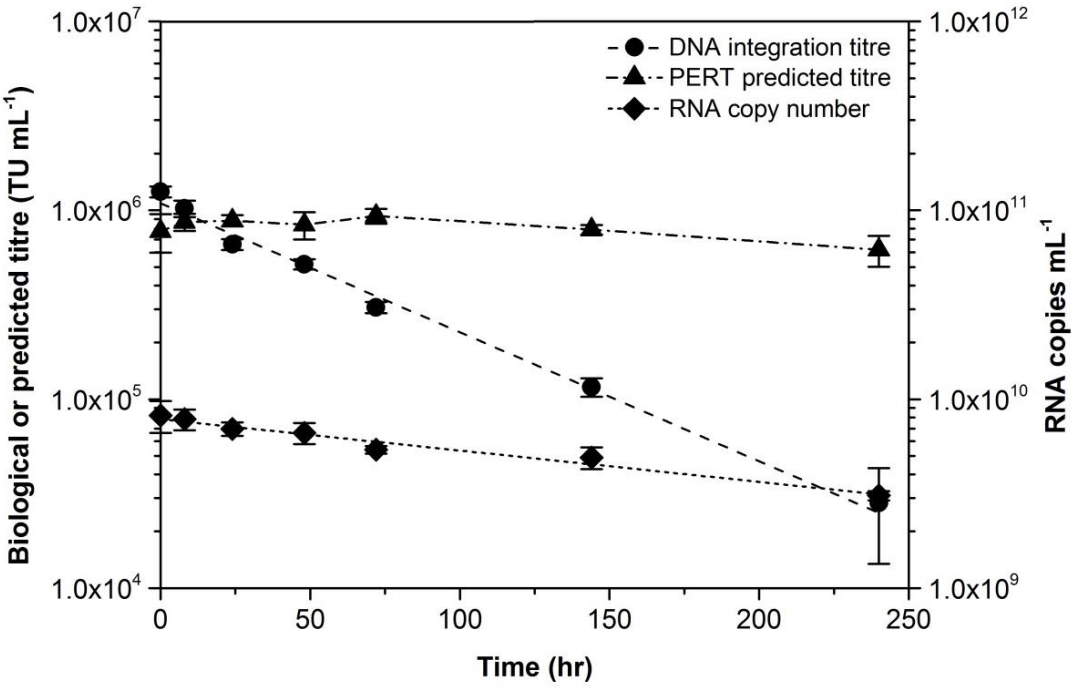
6.2.3.2 Lentiviral vectors encoding *eGFP* or a candidate therapeutic transgene

Lentiviral vectors expressing fluorescent marker genes, such as *GFP*, are commonly used for process development studies, as titres can be rapidly quantified using either microscopy or flow cytometry techniques. Use of a marker gene in place of a therapeutic transgene is only worthwhile, however, if the lentiviral vector production and decay kinetics are not altered by this substitution. It is known that certain therapeutic transgenes, such as factor VIII for the treatment of haemophilia A, can inhibit functional vector production unless their expression is diminished by use of a tissue-specific promoter (Radcliffe *et al.*, 2008). The ProSavin® transgenes (TH, AADC and CH1) are under the control of an internal cytomegalovirus (CMV) promoter (see Section 1.3.3, Figure 1.3 and Section 2.10.3, Figure 2.1), thus are likely expressed at reasonably high levels in producer cells. As it was unknown whether the stability of EIAV-derived lentiviral vector particles was affected by the particular transgene(s) expressed from the genome construct, the effect of switching the ProSavin® genome construct for one encoding either the marker gene *enhanced GFP* (*eGFP*), or an alternative candidate therapeutic gene was investigated. The eGFP vector and Vector 13.1 were generated via the transient transfection of adherent HEK293T cells using the same methodology, Gag/Pol and envelope plasmids as were earlier employed in the production of ProSavin® (Section 2.10.3). Crude supernatants containing the eGFP vector or Vector 13.1 were incubated at 36.5 °C for up to 240 or 96 hr, respectively. Again, the length of the incubation period was chosen such that a reduction in functional vector (here quantified using the DNA integration assay) to low, but not unquantifiable, levels could be observed (a full description of the methods employed is given in Section 2.10.1). To supplement functional titre data, PERT predicted titres and RNA copy numbers were also assessed.

The eGFP vector was found to be remarkably stable at 36.5 °C (Figure 6.20). Functional particles exhibited a half-life of 44.1 hr, while vector RNA displayed a half-life of 181.5 hr, and PERT predicted titres varied little throughout the incubation period of 240 hr. The decay kinetics were surprising, and completely unlike those observed for ProSavin® generated using the same means (Figure 6.19). Vector 13.1 was also found to be relatively robust in comparison to ProSavin®: functional particles degraded with a half-life of 33.7 hr, while vector RNA declined at a rate of 150.4 hr (Figure 6.21). PERT predicted titres, again, remained

consistent throughout the incubation period (96 hr). The differing half-lives were not correlated with size of the genome construct: although the eGFP expression plasmid (pONYKG) was the smallest (7,440 bp) of those evaluated, the Vector 13.1 expression plasmid (pONYK13.1) was the largest, and the ProSavin® expression plasmid (pONYK1) was of intermediate size (11,296 bp). To aid comparison of the various stability data obtained for the different EIAV vector preparations examined in this chapter, a summary table (Table 6.5) is included at the end of this section.

(a)

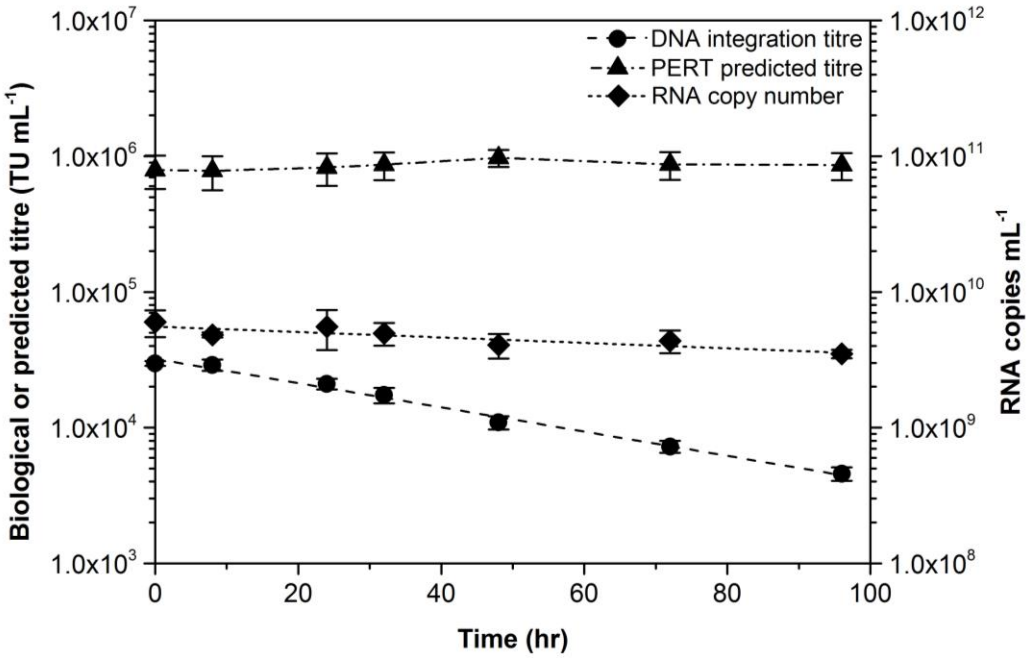


(b)

Measured response	Intercept (*a*)	Slope (*b*)	R^2	*p* value	$t_{½}$ (hr)
DNA integration titre	13.90 ± 0.12	-0.0157 ± 0.0011	0.979	< 0.0001	44.1 (41.2, 47.4)
RNA copy number	22.78 ± 0.07	-0.0038 ± 0.0007	0.889	< 0.0001	181.5 (155.5, 218.7)

Figure 6.20 Stability of functional eGFP vector, vector RT and RNA during the incubation of crude supernatant (generated by transient transfection using adherent cultures of HEK293T) at 36.5 °C. Biological (DNA integration) titres, PERT predicted titres and RNA copy numbers were quantified as noted for Figure 6.18 (full experimental procedures were as outlined in Section 2.10). Mean data is presented in plot (a) and error bars represent ± 1 SD (n = 3). Exponential trend line construction was completed according to the method detailed in Figure 6.1, using the results of linear regression analysis presented in table (b). Values were transformed by taking the natural logarithm prior to linear regression analysis. Slope and intercept values include a 95 % confidence interval. The half-life of functional eGFP vector and vector RNA (with upper and lower 95 % confidence limits) was calculated using Equation 6.1 as described in Section 6.2.1.1.

(a)



(b)

Measured response	Intercept (*a*)	Slope (*b*)	R^2	*p* value	$t_{½}$ (hr)
DNA integration titre	10.38 ± 0.08	-0.0206 ± 0.0015	0.976	< 0.0001	33.7 (31.3, 36.4)
RNA copy number	22.44 ± 0.14	-0.0046 ± 0.0027	0.409	0.0018	150.4 (95.3, 355.5)

Figure 6.21 Stability of functional Vector 13.1, vector RT and RNA during the incubation of crude supernatant (generated by transient transfection using adherent cultures of HEK293T) at 36.5 °C. Biological (DNA integration) titres, PERT predicted titres and RNA copy numbers were quantified as noted for Figure 6.18 (full experimental procedures were as outlined in Section 2.10). Mean data is presented in plot (a) and error bars represent ± 1 SD (n = 3). Exponential trend line construction was completed according to the method detailed in Figure 6.1, using the results of linear regression analysis presented in table (b). Values were transformed by taking the natural logarithm prior to linear regression analysis. Slope and intercept values include a 95 % confidence interval. The half-life of functional Vector 13.1 and vector RNA (with upper and lower 95 % confidence limits) was calculated using Equation 6.1 as described in Section 6.2.1.1.

Table 6.5 Summary of stability data for various EIAV vector preparations during incubation at 36.5 °C. Mean half-life ($t_{½}$) values are presented with upper and lower 95 % confidence limits (CL). Details of the particular methods employed and associated linear regression statistics may be obtained by referring to the relevant figure reference. Note that ProSavin® generated by transient transfection of adherent HEK293T exhibited two distinct inactivation phases; $t_{½}$ values for both phases are provided.

Measured response	Genome	Production method	Cell growth mode	Starting concentration (0 hr)	Incubation period (hr)	$t_{½}$ (hr) during stated incubation period			Figure Reference
						Mean	Lower CL	Upper CL	
ProSavin® titre	ProSavin®	Stable	Suspension	$2.6 \pm 0.2 \times 10^4$ TU mL^{-1}	0 - 30	10.1	9.4	10.8	6.4, 6.5 (f)
	ProSavin®	Stable	Adherent	$4.7 \pm 0.1 \times 10^4$ TU mL^{-1}	3 - 48	16.1	14.9	17.6	6.18
	ProSavin®	Transient	Adherent	$4.4 \pm 0.1 \times 10^5$ TU mL^{-1}	0 - 8	18.0	14.4	24.2	6.19
					8 - 24	2.4	2.3	2.6	
DNA integration titre	ProSavin®	Stable	Adherent	$7.3 \pm 0.5 \times 10^4$ TU mL^{-1}	0 - 48	21.2	18.6	24.8	6.18
	ProSavin®	Transient	Adherent	$7.2 \pm 0.6 \times 10^5$ TU mL^{-1}	0 - 8	21.5	15.3	36.0	6.19
					8 - 24	3.2	3.0	3.4	
	eGFP	Transient	Adherent	$1.3 \pm 0.1 \times 10^6$ TU mL^{-1}	0 - 240	44.1	41.2	47.4	6.20
	Vector 13.1	Transient	Adherent	$3.0 \pm 0.1 \times 10^4$ TU mL^{-1}	0 - 96	33.7	31.3	36.4	6.21
RNA copy number	ProSavin®	Stable	Suspension	$2.9 \pm 0.1 \times 10^9$ RNA copies mL^{-1}	0 - 48	23.2	21.0	26.1	6.6
	ProSavin®	Stable	Adherent	$2.0 \pm 0.2 \times 10^9$ RNA copies mL^{-1}	0 - 48	82.3	60.7	127.7	6.18
	ProSavin®	Transient	Adherent	$2.2 \pm 0.1 \times 10^{10}$ RNA copies mL^{-1}	0 - 12	29.6	19.8	58.9	6.19
					12 - 24	4.6	4.3	5.1	
	eGFP	Transient	Adherent	$8.2 \pm 1.6 \times 10^9$ RNA copies mL^{-1}	0 - 240	181.5	155.5	218.7	6.20
	Vector 13.1	Transient	Adherent	$6.0 \pm 1.3 \times 10^9$ RNA copies mL^{-1}	0 - 96	150.4	95.3	355.5	6.21
PERT predicted titre	ProSavin®	Stable	Suspension	$4.9 \pm 1.0 \times 10^5$ PERT predicted TU mL^{-1}	0 - 48	N/A - no decay observed			6.7 (a)
	ProSavin®	Stable	Adherent	$4.8 \pm 0.4 \times 10^5$ PERT predicted TU mL^{-1}	0 - 48	N/A - no decay observed			6.18
	ProSavin®	Transient	Adherent	$1.4 \pm 0.2 \times 10^6$ PERT predicted TU mL^{-1}	0 - 24	N/A - no decay observed			6.19
	eGFP	Transient	Adherent	$7.7 \pm 1.8 \times 10^5$ PERT predicted TU mL^{-1}	0 - 240	N/A - no decay observed			6.20
	Vector 13.1	Transient	Adherent	$7.9 \pm 2.2 \times 10^5$ PERT predicted TU mL^{-1}	0 - 96	N/A - no decay observed			6.21

6.3 Chapter discussion

Throughout the earlier microwell and WAVE bioreactor process development studies, described in Chapters 3 - 5, post-induction period was consistently observed to be a critical parameter impacting on ProSavin® titres. Titres declined during the latter stages of batch cultures, indicating that functional particles were inactivated in culture supernatants over time. The overall aim of this chapter was to characterise the stability of ProSavin® and other EIAV-derived lentiviral vectors, under typical production conditions, as well as to investigate various strategies to moderate or offset losses in functional particles, with a view to informing the design of improved upstream processes for EIAV-based lentiviral vectors.

6.3.1 The half-life of functional ProSavin® at 36.5 °C is short relative to the production timescale

Firstly, the decay kinetics of ProSavin® contained within crude supernatants derived from suspension cultures of PS46.2 incubated at 36.5 °C were examined. The majority (87 %) of functional ProSavin® was inactivated during the first 30 hr incubation (Section 6.2.1.1). Functional ProSavin® exhibited a half-life of 10.1 hr over this period (after 30 hr the rate of decay appeared to moderate slightly - observe that the 36 and 48 hr data points fall above the exponential trendline fitted to 0 - 30 hr data in Figure 6.4). This half-life value is within the range (1.3 - 10.4 hr) previously reported for functional HIV-1 derived lentiviral vectors pseudotyped with VSV-G incubated in crude supernatants at 37 °C (Higashikawa and Chang, 2001; Zhang *et al.*, 2004). The half-life of functional ProSavin®, though reasonably long in comparison to HIV-derived lentiviral vectors, is short relative to the overall duration of PS46.2 batch cultivations, and provides insight into why harvest timing is such a critical factor affecting titres. To gain insight into the mechanism of ProSavin® inactivation, the stability profiles of vector RNA and RT were also examined. During a 48 hr incubation period, vector RNA decayed with a half-life of 23.3 hr, while PERT predicted titres (based on a measurement of vector RT) did not decline. Later studies, conducted at 4 °C or using different EIAV vector preparations (Sections 6.2.2.1 and 6.2.3), also demonstrated that RNA copy numbers declined at a slower rate than functional titres, while PERT predicted titres did not reduce over the incubation periods investigated. Together, the data indicate that ProSavin® inactivation is unlikely to be caused by RNA or RT

degradation. It has recently been hypothesised that loss of the ability to perform reverse transcription is the main mechanism responsible for the inactivation of retroviral and HIV-1 derived vectors incubated at 37 °C (Carmo *et al.*, 2008; 2009b). Carmo *et al.* (2008) and (2009b) reported that two early stages of the reverse transcription process (initiation of DNA synthesis and first strand transfer) were particularly thermolabile however degradation of vector RNA or RT was not implicated as, consistent with our findings, these components were relatively stable at 37 °C. The thermosensitivity of these two stages, and overall inactivation rate of HIV-1 derived vectors, was affected by the choice of envelope protein (amphotropic or RDpro) (Carmo *et al.*, 2009b). It is worth bearing in mind, therefore, that the half-life of ProSavin® recorded here is likely also a function of the particular envelope protein (VSV-G) employed.

6.3.2 Autotransduction has a negligible impact on vector titres; cellular conditioning of the medium may be important for vector stability

Secondly, it was considered whether vector losses during upstream processing may be exacerbated due to the autotransduction of PS46.2 producer cells. Stewart *et al.* (2011) previously reported that, following four days (adherent) cultivation of induced PS46.2, the number of vector genome copies per cell increased from approximately one to seven, indicating that this cell line is susceptible to autotransduction over time (Stewart *et al.*, 2011). As theoretically this could represent a significant depletion in the number of functional particles available for harvesting, we investigated this phenomenon further. An EIAV-derived lentiviral vector, which expressed *LacZ* and could thus be quantified independently to ProSavin®, was incubated in the presence of microwell cultures of PS46.2 and, for comparison, in fresh medium not containing cells. It was discovered that, contrary to expectations, *LacZ* titres were diminished to a greater extent following 48 hr incubation in fresh medium, as compared to in the presence of PS46.2 (Section 6.2.1.2). It was hypothesised that the cells 'conditioned' the medium in a way that helped to preserve functional LacZ vector. This was explored further by incubating LacZ vector in medium that either had or had not been pre-conditioned (i.e. previously used for cell cultivation). The change in titre was assessed following a shorter incubation period (24 hr) and, although titres were slightly higher following incubation in the pre-conditioned medium, the results were not conclusive. As the benefit of medium conditioning

was slight in this second study, it is unlikely that this effect masked extensive losses of functional vector due to autotransduction in the first study, and overall it may be concluded that autotransduction is unlikely to significantly reduce ProSavin® titres during upstream processing – at least not directly. It is possible that autotransduction may reduce titres indirectly during extended cultivations by interfering with the genetic composition of producer cells, which could compromise cellular productivity over time, however this has not been investigated. The unexpected discovery that conditioned media can better preserve functional lentiviral vectors has implications for the future design of e.g., fed-batch or perfusion based production strategies, where the ratio of fresh to conditioned medium requires consideration.

6.3.3 Development of a perfusion strategy for ProSavin® manufacture could improve upstream yields

Three strategies to reduce ProSavin® inactivation during upstream process were tested: refrigeration of crude supernatants at 4 °C, reduction in the temperature at which ProSavin® is produced (to 31.5 °C), and elevation of the medium osmolality by the addition of fructose, glucose and/or sorbitol. These approaches were chosen following a review of published literature: storage of crude supernatants at 4 °C has been shown to significantly extend the half-life of HIV-1 derived vectors (Higashikawa and Chang, 2001), while a 5 °C reduction in production temperature (Kotani *et al.*, 1994; Lee *et al.*, 1996; Kaptein *et al.*, 1997; McTaggart and Al-Rubeai, 2000) or adjustment of the medium osmolality by sugar supplementation (Coroadinha *et al.*, 2006a; 2006b; 2006c) has been demonstrated to enhance retroviral vector titres, in part by increasing vector stability.

The half-life of functional ProSavin® at 4 °C was found to be approximately six days (Section 6.2.2.1). However, this rate only applied during the initial four days or so of storage at this temperature – after this point no further decay was observed when a time period of up to 16 days was examined, thus more than 50 % of functional vector was preserved over this period. This data is consistent with that obtained for murine leukaemia virus (retroviral) and HIV-1 derived lentiviral vectors pseudotyped with VSV-G, which have also been reported to exhibit two distinct phases of inactivation at both 37 and 4 °C (Higashikawa and Chang, 2001). Higashikawa and Chang (2001) suggested that these biphasic decay kinetics may indicate the

presence of two distinct vector populations within crude supernatants (one more fragile than the other), and this theory may be extended to EIAV-derived ProSavin®. Also synonymous with our data, HIV-1 derived vectors were reported to exhibit a substantially longer half-life at 4 °C as compared to during incubation at 37 °C: half-lives calculated for the second decay phase were around 200 *versus* 10.4 hr, respectively (Higashikawa and Chang, 2001). Overall, as short-term refrigeration of crude supernatants largely preserved ProSavin® functionality, this suggests a perfusion strategy where supernatants are continually removed to low temperature storage could improve overall yields obtained during upstream processing. A perfusion strategy has been previously applied successfully for the production of HIV-1 derived vectors in a stirred tank bioreactor (Ansorge *et al.*, 2009). However, this process was based on transient transfection of HEK293 cells using PEI (Ansorge *et al.*, 2009), and it remains for perfusion methods to be applied to the production of lentiviral vectors based on stable packaging or producer cells lines.

6.3.4 Reducing the culture temperature, or increasing the medium osmolality, did not confer any benefit for ProSavin® production

Further attempts to reduce ProSavin® inactivation and thus enhance titres during upstream processing were unsuccessful. Despite numerous reports that lowering the production temperature by 5 °C improved retroviral vector yields (Kotani *et al.*, 1994; Lee *et al.*, 1996; Kaptein *et al.*, 1997; McTaggart and Al-Rubeai, 2000), when applied to the ProSavin® process this approach severely compromised titres (maximum titres were around five-fold lower than those obtained from parallel cultures incubated at 36.5 °C; Section 6.2.2.2). The failure of this strategy to yield any positive benefit when applied to the production of ProSavin® likely arose from inherent differences between the vector systems employed (all published literature pertained to gamma-retroviral, rather than lentiviral, vectors).

As it had also been reported that retroviral vector titres could be enhanced by elevating the medium osmolality through the addition of fructose, glucose and/or sorbitol (Coroadinha *et al.*, 2006a; 2006b; 2006c), a study was also undertaken to test whether this strategy could confer any benefit for the ProSavin® process. However, of the nine different conditions evaluated, all produced a reduction in titres (Section 6.2.2.3). Again, the use of a different vector type (lentiviral as compared to gamma-retroviral) and a different production approach, likely

accounted for this result. As altering the osmotic properties of the medium through the addition of various sugar types affected ProSavin® titres so greatly (zero values were obtained for the most heavily supplemented conditions), this suggests that medium osmolality and/or sugar content are important process factors that possess an optimal range. The mildest conditions evaluated were those where the culture medium was supplemented with 100 mM fructose, glucose or sorbitol (note that the culture medium already contained glucose at a concentration of around 23 - 25 mM, see Section 6.2.2.3) and this raised the medium osmolality from 0.275 osmol kg^{-1} to 0.346 - 0.361 osmol kg^{-1}. This increase was perhaps too extreme, and it may be worthwhile for future studies to examine the effect of smaller adjustments. As the studies described by Coroadinha *et al.* (2006a), (2006b) and (2006c) employed an adherent cell line, they used a DMEM-based culture medium with a starting osmolality around 0.325 - 0.335 osmol kg^{-1}. It is therefore possible that the reported titre benefits were not replicated here due to the use of a different medium, and this is something else that could be investigated in the future. Finally, it may be that an acclimation period of three days was insufficient for the cells to adjust to the altered conditions, therefore the effect of culturing the cells for a longer period in the different media before evaluating titres could also be examined.

6.3.5 Different production methods and transgenes give rise to lentiviral vectors with distinct stability properties

The decay kinetics of ProSavin® when incubated at 36.5 °C were found to depend on the particular production system employed (Section 6.2.3.1). Functional ProSavin® derived from adherent cultures of PS46.2 exhibited a longer half-life (16.1 - 21.2 hr) than ProSavin® derived from suspension cultures of PS46.2 (Section 6.2.1.1). Functional ProSavin® derived from transiently transfected HEK293T cell cultures exhibited a decay profile characterised by two relatively discrete phases. Initially (between 0 and 8 hr), titres declined with a half-life of 18.0 - 21.5 hr, however subsequently (between 8 and 24 hr incubation) titres exhibited a half-life of 2.4 - 3.2 hr, and were consequently rapidly diminished. It is unclear why ProSavin® generated using the transient transfection process displays this unique biphasic stability profile, or what may be responsible for the rapid inactivation of functional vector post-8 hr incubation. Vector particles would have had a distinct composition to those generated using the stable producer cell line (PS46.2). Transfection reagents and plasmid DNA may have been co-packaged inside

vector particles along with, presumably, differing quantities of the vector transgene products, which may have made particles more susceptible to inactivation.

Switching the ProSavin® genome construct for one encoding either the marker gene *eGFP* or a candidate therapeutic gene (Vector 13.1) also greatly influenced the decay kinetics of vectors generated by transient transfection (Section 6.2.3.2). The eGFP vector was extremely stable (functional particles exhibited a half-life of 44.1 hr), and Vector 13.1 was also reasonably robust (functional particles displayed a half-life of 33.7 hr) when compared to ProSavin® (Section 6.2.3.1). All transgenes are encoded downstream of an internal CMV promoter (Section 2.10.3, Figure 2.1) and it is hypothesised that transgene proteins expressed in producer cells impacted on the stability properties of lentiviral vectors to a relatively greater or lesser degree. The half-lives of the eGFP vector and Vector 13.1 significantly exceed previously published half-life estimates for HIV-1 derived vectors incubated in crude supernatants at 37 °C (Higashikawa and Chang, 2001; Zhang *et al.*, 2004; Carmo *et al.*, 2009b). This could reflect differences in the cell lines and protocols employed for vector production. For example, the cholesterol content of producer cell membranes (which is affected by factors such as medium composition, temperature and, presumably, cell type) has been cited as an important factor determining the stability of budding retroviral vectors (Beer *et al.*, 2003; Carmo *et al.*, 2006; Coroadinha *et al.*, 2006c). It is also possible that lentiviral vectors derived from EIAV may be inherently more stable than those derived from HIV-1. The differing life cycles of the wild-type virus provide a potential insight into why this may be the case: EIAV is transmitted between hosts via an insect vector (thus has evolved to remain viable for certain periods external to the host), while transmission of HIV-1 between individuals relies solely on the direct exchange of bodily fluid (Narayan and Clements, 1989). In addition, wild-type EIAV is naturally exposed to higher temperatures than HIV, as the average core body temperature of a horse is around 38 °C (Green *et al.*, 2005), which is one degree higher than that of a human. Overall, the data indicate that the use of different production methods and vector genome constructs can have a considerable impact on resulting lentiviral vector decay kinetics, therefore optimisation of certain processing factors, particularly harvest timing, should be completed using individual vector systems.

7 Conclusions and future work

7.1 Conclusions

Lentiviral vectors used in clinical trials are currently produced by transient transfection of adherent HEK293(T) cells in multi-layered cell factories (Slepushkin *et al.*, 2003; Negré *et al.*, 2008; Schweizer and Merten, 2010; Merten *et al.*, 2011; Stewart *et al.*, 2011; Ausubel *et al.*, 2012). This approach cannot be scaled up, though scale-out is possible at considerable cost and logistical expense. For commercialisation the development of alternative strategies based on suspension-adapted stable producer cell lines is more desirable (Ansorge *et al.*, 2010; Schweizer and Merten, 2010; Segura *et al.*, 2013). To assist the development of such strategies, this thesis established a microscale cell culture platform that enables key bioprocess design data to be acquired rapidly and cost-effectively. The platform was used to define a scalable upstream process for ProSavin®, an EIAV-derived lentiviral vector developed for treatment of Parkinson's disease that has recently completed a Phase I/II clinical trial (Mitrophanous *et al.*, 1999; Azzouz *et al.*, 2002; Jarraya *et al.*, 2009; Stewart *et al.*, 2009; Stewart *et al.*, 2011; Palfi *et al.*, 2014).

The suitability of a shaken 24-well plate system for the suspension culture of HEK293T-derived producer cells, and its utility when combined with statistical DoE techniques for rapidly identifying key operating parameters impacting on lentiviral vector titre, was first established in Chapter 3. Initial evaluative studies demonstrated that the microwell system, operating with a 0.8 mL working volume, could support comparable cell growth, metabolite and pH kinetics as traditional shake flasks operating with a 50 mL working volume, when the HEK293T-derived dox-inducible ProSavin® producer cell line, PS46.2, and a second HEK293T cell line not stably transfected with vector DNA were examined (Sections 3.2.1 and 3.2.3). Lentiviral vector (ProSavin®) titres obtained from PS46.2 cultured in both vessel types were also comparable (Section 3.2.2). The data indicated that subsequent process development studies could feasibly be performed using the microwell system, yielding a 62.5-fold reduction in culture volume and substantially increasing the opportunity for parallelisation and thus experimental throughput. Six 24-well plates can be slotted on to a single clamp unit, enabling 144 experimental conditions to be investigated in parallel. The area occupied on a shaking platform

by the clamp unit is roughly equivalent to that of four conventional 250 mL shake flasks. Thus, a 36-fold increase in experimental throughput is enabled while the equipment footprint remains the same. For the microwell platform to be a truly effective bioprocess development tool, however, it was necessary to verify that any data generated was readily scalable. Although scale-up from microwells to WAVE bioreactors has been previously achieved on the basis of a single criterion, mixing time (Gill *et al.*, 2011), application of this approach to the ProSavin® process was found to be impracticable. The previous study by Gill *et al.* (2011) had focused on cell culture kinetics and antibody production using a CHO cell line, and the microwell mixing conditions used were more vigorous than those found to be optimal for PS46.2 cell culture and ProSavin® production in the current work. As a consequence of the gentle agitation conditions favoured by PS46.2, mixing times obtained in microwells were long and could not be replicated at large scale (Section 3.2.4), and alternative scaling criteria were consequently sought using DoE methodology. The DoE approach employed here comprised three key stages: (i) *screening*, (ii) *locating the optimal region*, and (iii) *optimisation* (Section 1.5.2, Figure 1.4). During the *screening* stage, a fractional factorial experiment was undertaken in microwells to examine the relative influence of nine processing parameters on ProSavin® titres obtained from PS46.2. The factors examined included biological variables (induction and harvest timings, serum concentration of medium, cell seeding density and inducer [dox and NaBu] concentrations) in addition to variables pertaining to the cell culture engineering environment (shaking speed, shaking diameter and liquid fill volume). Three parameters were identified as having a critical impact on titres. These were post-induction period (time to harvest), liquid fill volume and concentration of dox (Section 3.2.5). These parameters were therefore taken forward for further characterisation studies to optimise process performance and inform the design of a scale-up strategy.

In Chapter 4, a series of DoE-guided experiments were undertaken in microwells to *locate the optimum region* and *optimise* the operating ranges for post-induction period, liquid fill volume and concentration of dox. This approach was shown to be efficient for optimising the titre and P:I ratio of ProSavin® preparations. The investigations revealed that: (i) an optimal harvest time existed between approximately 26 and 46 hr post-induction, during which maximal titres of around 6×10^4 TU mL^{-1} could be attained, (ii) 1 µg mL^{-1} dox was sufficient for the full induction

of PS46.2 producer cells over this timeframe, and (iii) liquid fill volume should be maintained at or below 1000 µL as higher volumes compromised titres and the P:I ratio (Sections 4.2.1 and 4.2.3). Post-induction period and concentration of dox were critical parameters as expression of ProSavin® is tightly controlled by the TetR regulatory protein (Stewart *et al.*, 2009; Stewart *et al.*, 2011). ProSavin® production is initiated upon addition of dox to the culture medium. Full cellular induction is achieved by the addition of dox to saturating levels. Here, 1 µg mL^{-1} dox was observed to be sufficient for the full induction of PS46.2 for culture durations of up to 70 hr, as further daily additions of 1 µg mL^{-1}, or a higher starting concentration of 3 or 5 µg mL^{-1} did not enhance titres (Section 4.2.3). ProSavin® production is terminated likely as a result of cell death, which is possibly a consequence of nutrient limitations, such as a lack of glutamine or an accumulation of ammonia (Section 3.2.1.4), or due to an accumulation of VSV-G, as the cytotoxic properties of this viral envelope protein have been previously noted (Burns *et al.*, 1993; Yee *et al.*, 1994; Yang *et al.*, 1995; Ory *et al.*, 1996). ProSavin® exists only transitorily in culture supernatants as it is continually inactivated over time (Chapter 6). Consequently, the changing production kinetics and constant decay of ProSavin® throughout the culture period combine to make the post-induction period a critical parameter. Liquid fill volume was discovered to be an important parameter as it influenced the fluid mixing and oxygen transfer characteristics of the culture. Cultures with liquid fill volumes exceeding 1000 µL (29 % of the total volume of the well) exhibited reduced titres and worse P:I ratios, in conjunction with lower pH and higher lactate values (Section 4.2.1.4). It is probable that oxygen transfer was limited in these cultures and this triggered PS46.2 to increase the production of lactate, which accumulated and forced a reduction in the pH. Although the decrease in pH was relatively small - cultures with a fill volume above 1 mL yielded pH values of 6.7, while cultures with a fill volume less than or equal to 1 mL exhibited a pH of between 6.8 and 7.2 - this decrease was accompanied by a four- to six-fold reduction in ProSavin® titres (Section 4.2.1.4). This acidification may have compromised the productivity of PS46.2 cells and/or accelerated the rate of ProSavin® inactivation. The fluid hydrodynamics within microwells operating under optimised conditions were further characterised using the *Re*, *Fr* and *Ph* numbers, and this revealed that fluid flow was likely laminar and dominated by gravitational forces, leading to gentle mixing of the culture (Section 4.2.2). Together, the data indicated that mild agitation combined with adequate aeration was optimal for ProSavin® production in microwells.

In Chapter 5, the insights obtained during the microwell experiments described in Chapters 3 and 4 were used to inform scale-up of the ProSavin® process to a single-use 2 L WAVE bioreactor. Feasibility studies were first conducted to establish a basic operating protocol. An initial run, conducted using a 1 L fill volume, produced acidic conditions and low titres (Section 5.2.1.1). The earlier understanding derived from microwell data that low pH conditions can develop when fill volumes are too high, presumably as the supply of oxygen to cells is restricted, directed a second study in which the fill volume was reduced to 0.5 L (25 % of the total volume of the culture chamber). No culture acidification was observed during this run and titres were improved (Section 5.2.1.2). The maximum titre observed was 2.5 x 10^4 TU mL^{-1}, thus insights obtained at the microwell scale had been successfully employed to design a WAVE bioreactor process capable of delivering ProSavin® titres within two to three-fold of those observed in microwells. This represents a 585-fold scale translation of the process. A series of experiments were next conducted in which different rocking rates (6, 12, 18 and 24 rocks min^{-1}) and post-induction periods (17, 23, 41, 47 and 65 hr) were examined, to verify whether the key conclusions generated using the microwell system, i.e. that gentle agitation while ensuring adequate oxygenation, and a post-induction period of around 26 - 46 hr were optimal for ProSavin® production, held true following scale-up of the process (Section 5.2.2). As microwell experiments had demonstrated that a dox concentration of 1.0 to 3.0 µg mL^{-1} was sufficient for full cell induction over production timescales of up to 70 hr, this parameter was given a fixed value within this range. The experiments demonstrated that vigorous agitation (produced by a rocking rate of 24 rocks min^{-1}) or limited oxygen availability (produced by a rocking rate of 6 rocks min^{-1}) compromised titres, verifying the hypothesis based on microwell data that mild agitation while ensuring sufficient oxygenation is critical for ProSavin® production. Maximum titres were observed at 23 hr post induction during the 12 or 18 rocks min^{-1} runs, which was 3 hr earlier than the optimum harvest window predicted during the earlier microwell experiments; however this discrepancy was attributed to batch-to-batch variability in the cell stock. Overall, the microwell data correctly predicted the important parameters affecting ProSavin® titres in WAVE bioreactor cultures, therefore the microwell platform may be viewed as an effective tool in the future development of lentiviral vector bioprocesses. The hypothetical steps required to take the WAVE bioreactor process described in this chapter from research development stage to validated commercial application was considered in Appendix A.

The microwell and WAVE bioreactor data consistently demonstrated that post-induction period was a critical factor impacting on ProSavin® titres from suspension cultures of PS46.2 (Chapters 3 - 5). As titres declined during the latter stages of the culture, this indicated that functional particles were inactivated over time. The half-life of EIAV-based lentiviral vectors was not known and, as this knowledge could be used to further improve bioprocess design, the half-life of ProSavin® and other EIAV-based lentiviral vectors was assessed as described in Chapter 6, and several approaches to moderate the rate of decay were also trialled. It was discovered that the half-life of ProSavin® generated from suspension cultures of PS46.2 was 10.1 hr at 36.5 °C, and during a 30 hr incubation period 87 % of ProSavin® was inactivated (Section 6.2.1.1). Lowering the temperature to 4 °C preserved more than 50 % of functional vector over a period of up to 16 days (Section 6.2.2.1). This suggests that a perfusion strategy, where supernatants are continually harvested and refrigerated, could improve overall yields obtained during upstream processing. Attempts to improve ProSavin® titres from batch cultivations of PS46.2, based on either a reduction in the culture temperature to 31.5 °C or adjustment of the medium osmolality by the addition of sugars, proved unsuccessful (Sections 6.2.2.2 and 6.2.2.3). The stability of ProSavin® produced from adherent cultures of PS46.2 or using transient transfection techniques was also compared, and the impact of substituting the ProSavin® transgene for one expressing eGFP or an alternative therapeutic transgene examined. ProSavin® derived from adherent PS46.2 exhibited a half-life of 16.1 - 21.2 hr (depending on the titration method) at 36.5 °C, while ProSavin® obtained by transient transfection displayed a half-life of 18.0 - 21.5 hr during the first 8 hr incubation and a half-life of 2.4 - 3.2 hr thereafter. The eGFP vector and Vector 13.1 (both generated by transient transfection) exhibited half-lives of 44.1 hr and 33.7 hr, respectively. It was thus revealed that vector half-life is a function of the particular production method and transgene employed, highlighting the necessity for bioprocess development studies to be completed using individual vector systems (Section 6.2.3). Finally, regardless of the choice of production method or transgene, it was observed throughout these investigations that the RNA and reverse transcriptase (RT) associated with vector particles declined at a slower rate than functional particles (Sections 6.2.1.1, 6.2.2.1 and 6.2.3), which is consistent with findings for retroviral (Carmo *et al.*, 2008) and HIV-1 derived vectors (Carmo *et al.*, 2009b), and supports the theory that degradation of these components is not the primary cause of lentiviral vector inactivation. It has been hypothesised that a loss in the functionality of other key

components of the pre-integration complex (PIC), such as nucleocapsid proteins or primer tRNA, may instead be responsible (Carmo *et al.*, 2008; 2009b).

The microwell system used throughout this thesis delivered data that was fundamental in informing the design of a WAVE bioreactor process for a single cell line (PS46.2). This section concludes by considering (i) the overall predictive capabilities of the microwell system and (ii) the limitations of single cell line studies. Microwell titres were two- to three-fold higher than those obtained from optimised WAVE cultures, and this discrepancy likely arose as the microwell system is not an exact replica of the WAVE bioreactor, and therefore additional, unforeseen factors affected cell culture performance upon scale-up. For example, titres may have been reduced as a consequence of the severe cellular aggregation observed during most WAVE runs (Section 5.2.2.1), where clumps were much larger than those previously observed in microwells operating under optimised conditions (Figure 4.9 insert). The increased aggregation within the WAVE cultures may have been due to the unique hydrodynamic environment, caused by the different mode of mixing (i.e. rocking not rotating) and the increased volume of cells. The vessel geometry, plastic type and air flow system also make the WAVE bioreactor distinct from the microwell. The utility of the microwell system is therefore mostly limited to the early stages of process development, when it may be used efficiently to screen a large number of variables, and identify those which most affect titres and should be closely monitored during scale-up. Here, application of this approach to the ProSavin® process delivered important insights that enabled the fast development of a WAVE bioreactor process capable of delivering titres that were close to (within two- to three-fold of) those observed in microwells. Thus while it is important to verify key conclusions and refine the process at scale, the microwell system may be viewed as an important early stage process development tool.

This work identified fill volume, harvest timing and dox concentration as key factors affecting lentiviral vector production. When developing processes for other dox-inducible stable producer cell lines, the importance of these factors is likely to be universal, however it is important to acknowledge that the optimum ranges for these factors will likely differ. The development of stable producer cell lines typically involves multiple rounds of antibiotic selection and cloning by limiting dilution (e.g. Stewart *et al.*, 2009). Each cell line is therefore likely to possess unique cell growth characteristics and have a differing set of culture requirements. For example, it was

observed that the HEK293T-derived PS46.2 cell line consumed lactate after 73 hr cultivation, while a HEK293T cell line not stably transfected with DNA did not, which had implications for the culture pH (Sections 3.2.1.3 and 3.2.3.3). The choice of product will also have an impact as, for example, lentiviral vectors expressing different transgenes were shown to have distinct stability characteristics (Section 6.2.3.2), suggesting that the optimal harvest time for each product will be unique. Therefore, while this thesis has identified key factors affecting lentiviral vector production, the optimal ranges for these factors are likely to be cell line and vector specific. Furthermore, when developing lentiviral vector processes using alternative cell lines it may be prudent to re-examine those factors earlier dismissed as having a lesser impact (e.g. the agitation conditions). This may be achieved quickly and efficiently using the microwell-based process development approach presented in this thesis.

7.2 Suggestions for future work

Future work should endeavour to develop the ProSavin® process towards commercial application. Appendix A provides general discussion of the possible next steps required to scale-up and validate the WAVE bioreactor process presented in Chapter 5, and covers aspects of process design, qualification and the continual verification of commercial batches. In contrast, this section discusses in more detail the immediate next steps required to address specific concerns with the current process that were identified as a result of the investigations described in this thesis, and that must be dealt with before further scale-up of the process is considered. First, as titres obtained using suspension-adapted PS46.2 fell short of those ideally required for clinical application, the adaption of HEK293T cells to serum-free suspension growth and subsequent creation of new stable producer cell lines is recommended (note that it is anticipated that ProSavin® titres of at least 2×10^5 TU mL^{-1} will be required for commercial application, based on values reported for clinical trial batches; Section 1.4.1, Table 1.7). Second, also with a view to improving crude titres, further investigations are recommended to better understand and potentially control the factors leading to lentiviral vector inactivation. Third, to improve process understanding and facilitate greater process control, increased instrumentation of the WAVE bioreactor system is proposed. Fourth, to improve total vector yields from individual WAVE bioreactor runs, the evaluation of a perfusion strategy is suggested. Together this work should advance the design of a commercially viable

manufacturing process for ProSavin®, and help expedite the development of manufacturing processes for alternative lentiviral vectors.

7.2.1 Cell line and media development

ProSavin® titres were reduced following adaption of the adherent PS46.2 cell line to suspension growth (see Section 1.4.5). Subsequent process optimisation studies using this cell line (Chapters 3 - 4) successfully informed the design of a scalable WAVE bioreactor process for ProSavin® production (Chapter 5), however did not restore ProSavin® titres to those levels previously recorded for adherent PS46.2. It is possible that titres had been diminished during the suspension adaption process due to changes in the morphology and membrane properties of cells (Ansorge *et al.*, 2010), or vector production was inhibited by cellular aggregation in suspension cultures (Merten *et al.*, 2001). To address the first of these concerns, it may be necessary to derive new ProSavin® stable producer cell lines using HEK293T cells that have already undergone suspension adaption. To address the second of these concerns, suspension adaption and subsequent cultivation of HEK293T cells should ideally be performed using serum-free media. The viability of generating high-titre lentiviral vector producer clones from cells previously adapted to serum-free suspension growth has been earlier demonstrated (Broussau *et al.*, 2008).

7.2.1.1 Development of serum-free media

The tendency of HEK293 cells and their derivatives to aggregate when cultured in suspension has been well documented (Wurm and Bernard, 1999; Liu *et al.*, 2006; Zhao *et al.*, 2007) and the PS46.2 cell line was found to be no exception (Sections 3.2.5.2, 4.2.1.5 [see Figure 4.9 insert], and 5.2.2.1). PS46.2 were cultivated in the WAVE bioreactor using FreeStyle 293™ expression medium supplemented with 5 % tet-free FCS (Chapter 5). The tet-free FCS contains calcium ions (Ca^{+}; Section 3.2.5.2), which are known to induce aggregative behaviour (Peshwa *et al.*, 1993; Zhao *et al.*, 2007). Cellular aggregation is undesired as cells trapped in the centre of clumps can become non-viable (Peshwa *et al.*, 1993), reducing the overall productivity of the culture (Merten *et al.*, 2001). With regard to the PS46.2 cell line, titres may also be diminished as a consequence of dox not reaching cells in the centre of clumps, and/or due to ProSavin® being unable to escape into the supernatant. Switching to a serum-free

medium formulation has been reported to reduce cellular aggregation (Côté *et al.*, 1998). Furthermore, the complete removal of animal-derived components from the cell culture medium would be advantageous from a regulatory standpoint. It is therefore recommended that elimination of FCS from the culture medium is trialled here. In the event that ProSavin® titres from PS46.2 are not improved as a result of these investigations, adaption of the adherent HEK293T cell line to FCS-free media is also recommended, with a view to the future development of new stable producer clones (Section 7.2.1.2). Adaptation of cell lines to FCS-free media should be completed using a process of gradual serum reduction, such as previously described (Stevenson *et al.*, 2009; Guy *et al.*, 2013). FreeStyle 293™ expression medium without serum or any form of serum-replacement has been previously used for lentiviral vector production (Segura *et al.*, 2007; Witting *et al.*, 2012). However, if complete removal of serum proves difficult, the potential of chemically-defined animal origin-free substitutes, e.g. the lipid mixture L5146 (Sigma-Aldrich) previously used during lentiviral vector production by Ansorge *et al.* (2009), could also be explored. To enable the rapid characterisation of various media formulations and cellular responses (e.g. cell growth, viability, metabolite consumption and production), this work should be undertaken using the microwell platform (described in Chapters 3 and 4 of this thesis). To ensure the timely identification of potential scale-up issues, such as medium-plastic interactions, WAVE bioreactor cultivations should be performed using the most promising formulations.

7.2.1.2 Creation of ProSavin® producer cell lines

If adaption of the PS46.2 cell line to serum-free conditions is found not to increase titres to clinically relevant levels, the next step would be to take forward the best media formulations and HEK293T suspension clones and generate new ProSavin® stable producer cell lines, adapting the techniques previously described for adherent cultures (Stewart *et al.*, 2009). Again, application of the microwell system would be crucial for this work, as this would facilitate the screening of large numbers of clones, maximising the likelihood of identifying a high-titre candidate. Screening for vector production should be performed on the basis of biological titre, and use of the ProSavin® flow cytometry assay (Section 2.11.2.1) is recommended, as higher throughput may be achieved using this assay as compared to the DNA integration assay (Section 2.11.2.3). RNA copy number or PERT predicted titre measurements (Section 2.11.3)

should not be used as a substitute for measurements of biological titre, as the relationship between these parameters and titre was shown to be variable (Chapters 4 - 6). Vector production should be screened at several harvest points, as the critical impact of post-induction period on ProSavin® titres was earlier demonstrated (Chapters 3 - 5). The half-life of generated vectors should also be examined, as this was shown to differ depending on the specific production conditions employed (Chapter 6), and will impact on the design of batch/perfusion production processes. Again, it should be verified that the chosen clones are amenable to growth in the WAVE bioreactor.

7.2.1.3 Microcarrier culture of adherent ProSavin® producer cells

If neither of the above approaches is successful in yielding a suspension process that delivers commercially relevant titres, a third option may be to use microcarrier cultures of the adherent PS46.2 cell line. This option is less ideal, as microcarriers increase process complexity and cost, and can generate contaminants that are difficult to eliminate downstream (Section 1.4.2). This approach is scalable, however, and the use of microcarrier cultures to generate lentiviral vectors in a WAVE bioreactor has been previously demonstrated (Throm *et al.*, 2009; Greene *et al.*, 2012). If the adherent PS46.2 cell line were to be used however, the evaluation of alternative culture systems, particularly fixed-bed technologies that support higher cell densities, would be worthwhile. For example, the Integrity® iCELLis™ bioreactor by Pall Corporation uses a culture volume of just 25 L, yet reportedly provides the equivalent surface area of 3000 roller bottles (each 1700 cm^2) (Pall Corporation, 2014). This is comparable to 807 of the 10-layer Cell Factory™ systems (each 6320 cm^2) currently used for the production of clinical-grade lentiviral vectors (Table 1.7; Section 1.4.2). A fixed-bed bioreactor is also well-suited to perfusion culture, if such a strategy were to be pursued using the adherent PS46.2 cell line (the relevance of a perfusion strategy for ProSavin® production based on suspension-adapted PS46.2 is discussed in Section 7.2.4).

7.2.2 Further investigation into factors affecting lentiviral vector stability

Lentiviral vector inactivation during upstream processing is undesirable as it reduces crude titres. Future studies should seek to build on the insights obtained during Chapter 6, to better understand and potentially control the factors affecting lentiviral vector stability. First, it is

recommended that the impact of production method and transgene choice on lentiviral vector half-life (Section 6.2.3) is verified using additional batches of material. Second, experiments should be conducted to elucidate why some lentiviral vectors are more or less stable than others, for example, is it the expressed proteins or the genome RNA sequence that affects the stability of lentiviral vectors containing different transgenes? Third, based on the outcome of these investigations, methods to improve vector stability should be tested. For example, if the ProSavin® transgene proteins were found to negatively impact on vector stability, future studies may seek to develop methods to suppress their expression during upstream processing.

7.2.3 Greater instrumentation for enhanced process monitoring and control

The purpose of the process development work suggested in Sections 7.2.1 and 7.2.2 is to support the establishment of a cell culture process that can deliver commercially relevant titres. In parallel, it is recommended that the WAVE bioreactor system should be further developed to enable greater process monitoring and control. A robust control strategy is a fundamental requirement of bioprocess validation (see Appendix A). At present, the WAVE bioreactor system described in Chapter 5 provides limited opportunity for online monitoring or feedback control. Mixing parameters, such as the rocking rate, rocking angle, air flow rate and fill volume are set and altered manually if desired during a run. Dissolved oxygen was the only response parameter measured using an *in situ* probe, and only the last 10 min of data could be stored on the WAVEPOD module - therefore to obtain insight into the oxygen profile during cell cultivation, it was necessary to note down readings periodically. It is worth noting, however, that the rocking rate may be automatically adjusted, or automated oxygen blending introduced, to maintain dissolved oxygen levels within defined limits (General Electric Company, 2008c), and this facility may prove useful once the relationship between oxygen levels, agitation and ProSavin® titres is better understood.

Other pertinent culture parameters, such as pH, cell growth and vector titres were measured offline following manual sampling at various intervals, which were dictated in part by the length of the working day. *In situ* pH probes are available for the WAVE bioreactor (General Electric Company, 2008c). Use of an *in situ* pH probe (as compared to a pH indicator strip outside of the CO_2 controlled culture environment) should improve the precision of pH readings and reduce

the requirement for in-process sample collection (desirable as sample removal poses a risk of contamination). Automatic acid-base addition or automatic CO_2 adjustments to control the pH are also feasible (General Electric Company, 2008c), the utility of which should be explored. To the best of my knowledge, the WAVE bioreactor does not currently support automated sample removal, therefore some innovation would be required if sample collection for the measurement of cell growth characteristics and vector titre is desired outside of conventional working hours (alternatively overnight staffing will be required). The automated Cedex XS cell counter used in this work (Section 2.11.1.1) already enables rapid measurement of cell density and viability. A similarly rapid measurement of vector titre would be advantageous, but is unfeasible due to the necessary inclusion of a cell transduction step. It may be possible to rapidly estimate total particle numbers, however, using a virus counter such as the Virus Counter 3100 developed by virocyt, and this data could be useful if found to correlate well with functional titre values. This information could assist the development of enhanced process control strategies, enabling harvest timing, for example, to be determined on a batch-by-batch basis. Another option would be to investigate the utility of technologies such as permittivity measurements for the real-time detection of vector release, the potential of which has been recently demonstrated (Ansorge *et al.*, 2011).

Lastly, documentation is an essential element of process validation that supplies the evidence that robust process control has been, and continues to be, achieved (European Commission, 2001; FDA, 2011). To accomplish this, a system to capture and retain inline and online data throughout the cultivation period needs to be established. The WAVEPOD has an Ethernet port which may be configured for external data collection (General Electric Company, 2008c), thus the utility of this feature should be explored.

7.2.4 Evaluation of a perfusion strategy

The final recommendation for future work is the evaluation of a perfusion strategy for ProSavin® production. ProSavin® generated using suspension cultures of PS46.2 was continually inactivated at 36.5 °C with a half-life of around 10.1 hr (Section 6.2.1.1). As the half-life of ProSavin® was greatly extended when supernatants were stored at 4 °C (more than 50 % was preserved for up to 16 days; Section 6.2.2.1), it is conceivable that overall yields may be

improved if a perfusion system were to be developed that enabled the continual harvest and refrigeration of culture supernatants. The simultaneous dilution of cytotoxic vector proteins (e.g. VSV-G) and metabolic by-products, and the replenishment of limiting nutrients, could also extend the productivity of the cell culture. The first goal would be to determine the value of this approach, i.e. to compare the increase in overall yield (titre x total volume) with that obtained from a batch cultivation. A perfused system will generate a bulk harvest that is larger but more dilute, and it should be ascertained whether the increase in overall yield is substantial enough to warrant the increased pressures imposed on the downstream processing steps as a result. Note that purification of ProSavin®-containing supernatants is currently undertaken prior to concentration (Section 1.4.3, Table 1.10), thus the cost implications and overall feasibility of purifying larger volumes of more dilute material should be considered. It is also worth noting that as culture duration increases, more of the cells will die, releasing DNA, host cell proteins and waste metabolites that also need to be removed downstream, which may add to the processing time and cost. In order to make a judgement as to whether a perfusion strategy is likely to be viable, a process that is reasonably optimal needs first to be developed. The microwell platform may serve as a rough mimic of the large scale process and assist the development of such a strategy, although the addition of media and harvesting of supernatants (ideally through a filter to prevent the removal of cells) could not be performed continually, and would need to be performed manually at regular intervals. The designed process should be refined using the laboratory scale WAVE bioreactor system.

Appendix A Bioprocess scale-up and validation

A.1 Introduction and aims

"Process validation" is the term used to describe the documented evidence, obtained from the initial process design stage through to commercial application, that a process can consistently deliver a product that meets predefined specifications (European Commission, 2001; FDA, 2011). Process validation is a prerequisite for regulatory approval for the commercial manufacture of biopharmaceuticals in Europe and the U.S. (European Commission, 2001; FDA, 2011). Chapters 3 to 5 of this thesis described the microwell characterisation and scale-up verification of an upstream processing route for ProSavin®, an EIAV-derived lentiviral vector. This appendix considers, hypothetically, the steps required to take the WAVE bioreactor process described in Chapter 5 from research development stage to validated commercial application. The specific objectives of this chapter are:

- To propose a strategy for refinement of the process and scale-up to commercial scale
- To review the procedures required for qualification of the commercial process
- To consider how continual process verification may be accomplished

A.2 Proposed approach to bioprocess scale-up and validation

A successful validation programme essentially depends on the detection, understanding and control of sources of process variability so that consistent product quality is assured (FDA, 2011). Process validation activities may be broadly divided into three stages: (1) process design, (2) process qualification, and (3) continued process verification (FDA, 2011). Below, each stage is briefly introduced and activities to support validation of the ProSavin® process are suggested. Throughout all stages, it is recommended that an enhanced, Quality by Design (QbD) approach is adopted. A detailed comparison between a typical minimal approach, and a QbD approach to process validation is provided in Appendix I of ICH (2009). Key aspects of a QbD approach include: (i) use of multivariate (rather than OFAT) experimental techniques to improve process knowledge, (ii) a manufacturing process that is not fixed, but is adjustable within the design space (iii) incorporation of process analytical technologies (PAT) for

responsive process control, and (iv) continual process improvement throughout the lifecycle of the product (ICH, 2009).

A.2.1 Process design

The goal of the process design stage is to use information obtained during small scale process development and scale translation studies to define a commercial manufacturing process (FDA, 2011). The commercial process should comply with current Good Manufacturing Practice (GMP) regulations, i.e. be designed and controlled to ensure that raw materials and the final biopharmaceutical product consistently meet predefined quality specifications (FDA, 2011).

As described earlier (Chapters 3 - 5), microscale bioprocess development studies led to the creation of a WAVE bioreactor process for ProSavin® production based on suspension cultures of a stable producer cell line (PS46.2). This process differs from that used to generate Phase I/II clinical material, and a comparison of the two approaches is provided in Table A.1. When seeking regulatory approval, significant differences between the manufacturing process used to generate material for clinical trials, and the process proposed for commercial application, require discussion, and the influence on the quality of the product understood (ICH, 2009). It would therefore be desirable to demonstrate, by reference to historical data, that the quality of ProSavin® was not affected by the changes to the upstream processing route proposed in this thesis. Quality is assured by maintaining key characteristics of the product, termed critical quality attributes (CQAs), within predefined specifications (ICH, 2009). Examples of CQAs for ProSavin® that may be assessed post-harvest (i.e. using crude supernatants) are listed in Table A.2. Titre is arguably the most important CQA, as this represents the concentration of functional vector particles, and is the primary indicator of quality used to guide bioprocess development studies. PERT and RNA copy number measurements are also important however, as this information may be used in conjunction with titre data to estimate the proportion of lentiviral vector particles that are functional, and thus provide insight into the overall quality of a given preparation. ProSavin® titres obtained when using the WAVE bioreactor process are approximately one to two orders of magnitude lower than those obtained from the current clinical GMP process (Table A.1). This issue must be resolved before further scale-up of the WAVE bioreactor process is considered.

As well as demonstrating that the post-harvest CQAs (Table A.2) are not affected by the change in the upstream manufacturing process, bridging studies may also need to be performed to demonstrate that the safety and efficacy of the product remains unaffected. This may include non-clinical toxicology and biodistribution studies in relevant animal models, and may also include further clinical studies. The scope and extent of this work should be decided following consultation with the appropriate regulatory authority.

Table A.1 Comparison of the current clinical GMP upstream process for ProSavin® production with the WAVE bioreactor process described in Chapter 5 of this thesis. Dox is an abbreviation for doxycycline, DMEM for Dulbecco's Modified Eagle's Medium, and FCS for foetal calf serum. *A 200 L vessel represents the proposed commercial scale, note that a 2 L WAVE bioreactor with 0.5 L fill volume was evaluated in this project. **Different quantitation methods were used to assess crude titres - GMP titres were determined using the DNA integration assay (Section 2.11.2.3), while WAVE bioreactor titres were determined using flow cytometry (Section 2.11.2.1). Earlier data (Section 6.2.3.1 of this thesis) indicated that titres quantified using flow cytometry may be around 40 % lower than those quantified using the DNA integration assay (compare corresponding starting concentration values in Table 6.5).

	Current GMP process	**Process evaluated in this thesis**
Reference	Schweizer and Merten (2010); Stewart *et al.* (2011)	Section 5.2.2 of this thesis (18 rock min^{-1} condition)
Expression system	Triple plasmid transient transfection of HEK293T cells using Lipofectamine™ 2000 CD reagent	Dox inducible producer cell line (PS46.2) derived from HEK293T with all vector components stably integrated
Cell growth mode	Adherent	Suspension
Culture medium	DMEM supplemented with 2 mM L-glutamine, 1 % non-essential amino acids and 10 % FCS	FreeStyle™ 293 expression medium supplemented with 5 % tetracycline-free FCS
Vessel	10-layer Cell Factory™	200 L WAVE bioreactor*
Production scale (L)	72 (24 per campaign)	50*
Crude titre (TU mL^{-1})**	0.2 - 2.0 x 10^6	2.4 - 4.1 x 10^4

Table A.2 Examples of critical quality attributes (CQAs) for ProSavin® that may be monitored post-harvest (prior to downstream processing and product formulation steps). Clarification refers to the low speed centrifugation and 0.45 µm filtration of supernatants to remove cells and cellular debris. The example specification for DNA integration titre is based on the lower limit of the reported range obtained for current clinical GMP batches (Table A.1). The example specification for ProSavin® titre as assessed by flow cytometry reflects the observation that titres quantified using this method may be around 40 % lower (Table A.1). Tests for mycoplasma, bioburden, endotoxin and adventitious agents should be conducted according to procedures outlined by the European Pharmacopoeia. Replication competent lentivirus (RCL) assays for EIAV-derived vectors have been described by Miskin *et al.* (2006) and Farley *et al.* (2012), while methods for quantifying functional titres, PERT predicted titres and RNA copy numbers were described in Sections 2.11.2.1, 2.11.2.3 and 2.11.3 of this thesis.

Step	Test	Specification
Crude supernatant (prior to clarification)	Mycoplasma	≤ *x*
	Bioburden	≤ *x*
	Endotoxin	≤ *x*
	Adventitious agents	≤ *x*
	replication competent lentivirus (RCL)	Negative
Crude supernatant (post-clarification)	DNA integration titre	≥ 2.0×10^5 TU mL^{-1}
	ProSavin® titre assessed by flow cytometry	≥ 1.2×10^5 TU mL^{-1}
	PERT predicted titre	None
	RNA copy number	None

A recommended strategy for defining a commercial manufacturing process for ProSavin® is outlined in Table A.3. The proposed strategy is based on the insights obtained during the early phase bioprocess development work described in this thesis (Chapters 3 - 6). In brief, it is recommended that initial studies should use the microwell system described in Chapters 3 and 4 to tackle the issue of low titres and cellular aggregation. Towards this aim, the evaluation of serum-free media formulations and the screening and selection of new stable producer cell lines is suggested. Following identification of a suitable medium formulation and cell line, it would be prudent to re-evaluate the relative impact of various processing parameters on ProSavin® titres. This work should incorporate Design of Experiments (DoE) techniques and may initially be conducted using the microwell system, although it is recommended that operating limits for important parameters should be refined and ultimately defined using a laboratory scale WAVE bioreactor that better mimics the proposed commercial scale vessel (a 200 L WAVE bioreactor). This work should inform the implementation of appropriate control strategies to assure consistent product quality. Finally, scalability between the laboratory and commercial scale

vessels should be verified. For the development of this strategy, it was assumed that the CQAs monitored post-harvest are those outlined in Table A.2, and that assays to quantitate these responses are validated. As the focus is on upstream process development it was also assumed that downstream processing steps are such as those currently employed for the purification of clinical GMP batches (e.g. see Section 1.4.3, Table 1.10) and these have also been validated. In reality, however, it is likely that certain changes, such as the removal of serum from the medium, will force modifications to be made to the downstream procedure, and this is something that should be investigated in parallel. Note that if titres are not improved as a consequence of the cell line and media development work proposed here (Table A.3, Activities 1 and 2), the feasibility of increasing the downstream concentration factor (which is currently 2000-fold, see Section 1.4.3, Table 1.10) should be investigated. It was noted in Section 1.4.5 that compensating for low crude titres by altering the downstream process was undesirable, as the final product would comprise a greater proportion of impurities. However, this approach may become viable if ProSavin® is generated using a stable producer cell line cultured in FCS-free media, as the starting material would contain fewer contaminants than that derived from the current clinical process (Table A.1), and the co-concentration of plasmid DNA, transfection reagents and animal-derived components would no longer present a concern.

Table A.3 Suggested strategy for defining a commercial manufacturing process for ProSavin®. Activities are broadly listed chronologically, although in practice some overlap between activities is expected. Initial operating conditions for microwells and the laboratory scale (2 L) WAVE bioreactor should reflect those described in Sections 2.4.5 and 2.7.3.3 (18 rocks min^{-1} condition) of this thesis, respectively.

Activity (scale)	Description
1. Adapt HEK293T cells to serum-free suspension culture (microwells)	It is desirable to eliminate FCS from the cell culture medium for two reasons. Firstly, from a processing perspective, it would be advantageous to diminish the calcium content of the medium so that cellular aggregation is reduced. Note that, in microwell cultures of PS46.2, aggregation was found to be exacerbated when higher concentrations of FCS (5 % *versus* 1 %) were used (Section 3.2.5.2), and in WAVE bioreactor cultures supplemented with 5 % FCS, cellular aggregation was extensive (Section 5.2.2.1). Eliminating FCS from the culture medium should yield a more homogenous cell population and possibly improve titres. The potential presence of extraneous agents in FCS provides a second reason why, from a regulatory perspective, it is preferable to eliminate this component from cell culture media (EMA, 2013). Lentiviral vector production has been achieved using FreeStyle 293™ expression medium without serum or any form of serum-replacement (Segura *et al.*, 2007; Witting *et al.*, 2012). It is preferable to continue to using FreeStyle 293™ expression medium for ProSavin® production as this medium has been used effectively throughout the bioprocess development work described in this thesis. This medium is chemically defined, free from animal-derived components and manufactured in a GMP compliant facility (Thermo Fisher Scientific, 2014). Although its formulation is proprietary, if the medium is to be used in a commercial manufacturing process it is possible to request access to the Drug Master File (which includes formulation information) that is lodged with the U.S. Food and Drug Administration (FDA). To adapt HEK293T cells to serum-free medium, a method of gradual serum reduction is recommended, such as previously described (Stevenson *et al.*, 2009; Guy *et al.*, 2013). If complete serum removal is unsuccessful, the use of animal origin-free substitutes could be explored. For example, the lipid mixture L5146 (Sigma-Aldrich) has previously been used successfully as a serum substitute during lentiviral vector production (Ansorge *et al.*, 2009) and its potential could be evaluated here. This work should be conducted using the microwell system, as this would enable multiple media formulations and cellular adaptation strategies to be evaluated. Promising formulations should be verified using a laboratory scale (2 L) WAVE bioreactor to facilitate the early identification of any potential scale-up issues, such as medium-Cellbag interactions.

2. Generate ProSavin® producer cell lines (microwells)	Titres were diminished five- to twenty-fold following the adaption of adherent PS46.2 to growth in suspension (Section 1.4.5). As subsequent process optimisation studies did not yield significant improvements in titre (Chapters 3 - 4), it is hypothesised that cellular aggregation and/or changes in the morphology and membrane properties of cells during adaption may have compromised titres (Section 1.4.5). Broussau *et al.* (2008) previously described the generation of high-titre lentiviral vector producer clones from cells that were already adapted to serum-free suspension growth. It is suggested that value of this approach is trialled here. ProSavin® stable producer cell lines should be generated as described previously (Stewart *et al.*, 2009), except that HEK293T cells already adapted to serum-free suspension growth should be used (see Activity 1.). At this stage, the possibility of transgene suppression during ProSavin® production could also be explored, as it is hypothesised that expression of the ProSavin® transgene cassette may limit vector half-life during upstream processing (Section 6.3.5). The microwell system should be employed to facilitate the screening of large numbers of clones and improve the chances of identifying high-titre candidates (the best candidates should be verified using the laboratory scale WAVE bioreactor). The developed cell lines and associated ProSavin® vector should be thoroughly characterised in terms of identity and genetic stability using methods such as those previously described (Stewart *et al.*, 2011). Note that, if titres are not improved as a consequence of the media and cell line development work suggested here (Activities 1 and 2), studies to investigate the feasibility of increasing the downstream concentration factor should be instigated and conducted in parallel to the further upstream process development work outlined below (Activities 3 to 6).
3. Establish critical processing parameters (CPPs) and their approximate optimal ranges (microwells)	Having identified a suitable medium formation and candidate ProSavin® producer cell line, it would be prudent to re-assess the impact of certain processing parameters on titre. This work should incorporate DoE techniques and may initially be conducted using the microwell system. Risk assessment tools, such as Ishikawa (fishbone) diagrams and failure mode effects analysis (FMEA) techniques, can be used to identify parameters for inclusion in DoE studies (ICH, 2009; FDA, 2011). The microwell studies conducted using the PS46.2 cell line (Chapters 3 and 4) should provide the starting point for this work. As well as identifying and establishing approximate ranges for critical processing parameters (CPPs), i.e. parameters whose variability impacts on a CQA and thus require monitoring and control to ensure consistent product quality (ICH, 2009), it would be desirable at this point to identify raw materials or process stages that may be eliminated without compromising CQAs. This would streamline the process, enable cost-savings, and ease regulatory approval. An example of a raw material that could potentially be removed is sodium butyrate (NaBu), as this was found to have no significant impact of ProSavin® titres obtained from PS46.2 over the range 0.2 - 1.8 µg mL^{-1} (Section 3.2.5.1).

4. Verify microwell findings and execute further studies to identify 'WAVE bioreactor specific' CPPs (laboratory scale WAVE bioreactor)	Operating ranges for CPPs should be verified using a laboratory scale WAVE bioreactor, which better mimics the commercial vessel. In addition, some processing parameters are 'WAVE bioreactor specific', such as air flow rate, and cannot be evaluated using the microscale system, therefore further risk assessment activities and DoE-based experimentation using the laboratory scale WAVE bioreactor will be necessary to establish operating ranges for these factors. Additionally at this stage (if not before), it would be worthwhile confirming that all raw materials to be used in the commercial process (including disposable plasticware, for example) conform to GMP specifications and are qualified for use in the generation of a medicinal product. The long-term supply of these materials should also be confirmed. It may also be necessary to conduct studies to demonstrate that plastic leaching from the disposable culture chamber does not present a concern.
5. Define process control approach (laboratory scale WAVE bioreactor)	Once the design space, i.e. the combination of raw material attributes and process parameters known to assure product quality (ICH, 2009), has been characterised, a control strategy to ensure that the process does not deviate from operation within the design space should be defined. Process control strategies may be designed to reduce input variation, adjust for input variation, or both (FDA, 2011). For example, this may be achieved by specifying fixed ranges for CPPs in the former instance, or by employing process analytical technology (PAT) in the latter. PAT affords increased process flexibility, as online analysis of important responses and the use of feedback loops can adjust the processing conditions to ensure the output remains constant (FDA, 2011). Harvest timing was demonstrated to be a critical parameter affecting ProSavin® titres from PS46.2, and the optimal time to harvest varied between batches (Chapters 3 - 5). To reduce titre variability as a consequence of shifts in the optimal harvest time, the potential of technologies such as *in situ* permittivity measurements for real-time detection of lentiviral vector release could be investigated (Ansorge *et al.*, 2011).
6. Scale-up to commercial size vessel (laboratory to commercial scale WAVE bioreactor)	It is acceptable for a design space to be developed at any scale, however the relevance of the design space should be justified, and potential risks in the scale-up operation understood (ICH, 2009). Verification that the design space and control strategy developed using the laboratory scale WAVE bioreactor is predictive of commercial scale process performance is therefore vital. As turbulence and oxygen availability were important factors affecting ProSavin® titres obtained from WAVE bioreactor cultures of PS46.2 (Chapter 5), Re_{mod} (Eibl and Eibl, 2006; Eibl *et al.*, 2010b) may be an appropriate parameter on which to base scale-up studies. Predictive scale-up of cell culture processes to 200 L WAVE bioreactor scale has been previously performed using this criterion in conjunction with knowledge of the impact of aeration rate, rocking conditions and liquid fill volume on oxygen transfer efficiency (Eibl *et al.*, 2010b). It will also be necessary to establish effective protocols for seed expansion, although this is not included in the design space and does not form part of the registered detail (Osborne, 2011), and procedures for representative sampling of commercial batches (FDA, 2011). Finally, the overall compliance of the commercial process design with GMP regulations should be demonstrated (European Commission, 2001).

A.2.2 Process qualification

A.2.2.1 Overview of process qualification

During the process qualification stage, the designed process is assessed to determine whether it is capable of reproducible commercial manufacture (FDA, 2011). This stage comprises two key elements: (i) facility design and qualification of utilities and equipment and (ii) process performance qualification (PPQ), which, if successful, should ultimately culminate in regulatory submission (FDA, 2011).

Proper design of the manufacturing facility is a requirement of GMP (FDA, 2011). New or modified utilities and equipment require proper qualification, and this typically includes the following activities (European Commission, 2001; FDA, 2011):

- Selecting construction materials, operating methods, and performance characteristics for utilities and equipment that are appropriate for their intended application
- Verifying that installed utility systems and equipment conform to the design specifications
- Verifying that utility systems and equipment operate in line with the requirements of the process over all expected operating ranges

PPQ succeeds the successful qualification of the facility, utilities and equipment. This step combines these elements with the trained personnel and designed process to produce commercial batches of material (FDA, 2011). The overall goal is to demonstrate that the commercial manufacturing process performs as expected (FDA, 2011), and typically three successful consecutive batches are considered sufficient for validation of the process (European Commission, 2001).

It is difficult to propose process qualification activities for ProSavin® manufacture without the process design stage being near complete. However, assuming the process design strategy proposed in Section A.2.1 (Table A.3) has been broadly followed and a 200 L WAVE bioreactor process developed, the following general considerations (Sections A.2.2.2 and A.2.2.3) may play a role in informing the preparation of a process qualification plan.

A.2.2.2 Process qualification in relation to ProSavin®: Facility design and qualification of utilities and equipment

The size of the facility should be based on projections regarding the number of patients to be treated each year, the dosage required for each patient, the shelf-life of the final product, and economic constraints. It may be desirable to conduct single runs consecutively using a small facility and a limited number of personnel, or it may be preferable to generate large volumes of material quickly using several bioreactors operated in parallel, although this would require bigger premises and more operating staff. As ProSavin® is one of a pipeline of products developed by Oxford BioMedica that are based on the EIAV lentiviral vector platform (Section 1.3.3) the facility, utility systems and equipment may need to be designed with a view to their subsequent/simultaneous application for the manufacture of several products. As well as the upstream processing area, the facility will need to include areas for cell expansion, downstream processing and product formulation. The overall layout of the facility should take into account whether one or more discrete processing suites are required for concurrent production of multiple batches of ProSavin® or other pipeline products.

A.2.2.3 Process qualification in relation to ProSavin®: PPQ

Prior to performing PPQ it would be prudent to verify the robustness of the commercial process several times by undertaking runs that test the extreme operating limits of the system (European Commission, 2001). PPQ may then be achieved by the production of three consecutive batches of ProSavin® material using the commercial process. As well as end-product testing, relevant CQAs should be monitored in-process (e.g. directly after vector harvests; Table A.2). Samples should be representative of the entire batch, and ideally an increased number of samples should be collected than would typically be during a commercial run. Successful validation will depend on whether the process specifications (including cleaning procedures) and product specifications are met for all batches.

A.2.3 Continued process verification

The aim of continued process verification is to monitor the process during routine commercial production to assure a continual state of control (FDA, 2011). Systems should be in place for

the collection and review of data during standard commercial runs, to enable the timely detection and resolution of undesired process variability (FDA, 2011). The commercial upstream process for ProSavin® may be continually verified by monitoring cell culture performance parameters and CQAs during each run using *in situ* probes and by regular offline sampling. Assuming a batch protocol were developed, the production timescale is likely to be short, around three or four days, thus to gain reasonable insight into the process performance samples for offline analysis would need to be taken and analysed throughout the day and night – necessitating staff to work in shifts. Alert systems should be in place in case immediate corrective action is needed to retain monitored parameters within set limits. At the end of each run, all data should be passed on to a team of trained statisticians, who should trend and review the data, highlighting areas for improvement. Continual improvement is as important as continual verification, and process failures should be assessed to inform the design and implementation of preventative strategies. Knowledge obtained through the analysis of commercial batch data and from parallel small scale experiments should also inform process changes that increase robustness or reduce costs. In addition, as much of the regulation pertaining to the manufacture of viral vectors for human use has not been formalised, the manufacturing process should have the capacity to incorporate changes driven by changing regulatory requirements.

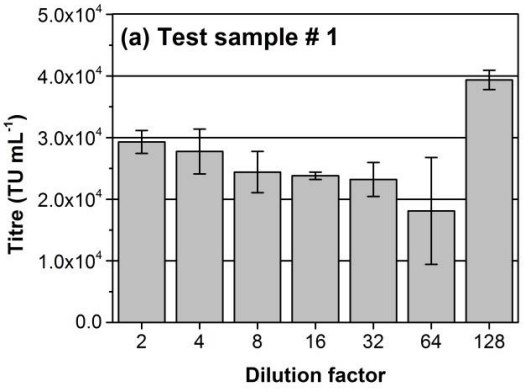
A.3 Summary

The purpose of this section was to consider how the WAVE bioreactor process described in Chapter 5 may be taken forward from research development stage to validated commercial application. Currently this process is in the relatively early stages of development. A strategy for refinement and scale-up of the process was proposed (Section A.2.1). It was suggested that the microwell platform described in Chapters 3 and 4 of this thesis could be usefully employed for initial media development and cell line selection studies, with a view to identifying a high-titre stable producer clone suited to growth in serum-free conditions. Subsequent studies using the microwell platform in conjunction with DoE techniques should ascertain the critical processing parameters (CPPs) and raw material attributes that impact on ProSavin® critical quality attributes (CQAs), in particular titre. The microwell findings should be verified using a laboratory scale 2 L WAVE bioreactor, and the impact of further WAVE bioreactor-specific parameters

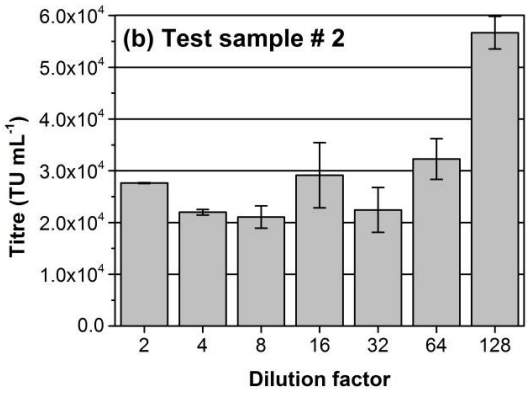
(e.g. air flow rate) examined. Operating limits and raw material specifications should ultimately be defined using this system and appropriate control strategies put in place to ensure consistent product quality. Knowledge of the effect of air flow rate, liquid fill volume, and rocking conditions on the turbulence and oxygen transfer capacity within different sized vessels should inform scale-up of the process to a 200 L vessel. Following the successful development of a GMP-compliant commercial process, the process requires qualification (Section A.2.2). Initially facilities, utilities and equipment should be chosen, installed and demonstrated to be working correctly and in line with design specifications. Subsequently these components may be combined with the personnel and manufacturing process, and process qualification completed by the successful demonstration that the process performs as expected during the production of three consecutive batches of material. Following the attainment of regulatory approval, continual verification of the commercial process should be performed by the collection and timely analysis of data obtained during commercial runs, leading to improvements in process control where necessary (Section A.2.3).

Appendix B Establishment of a LOQ for the ProSavin® titre assay

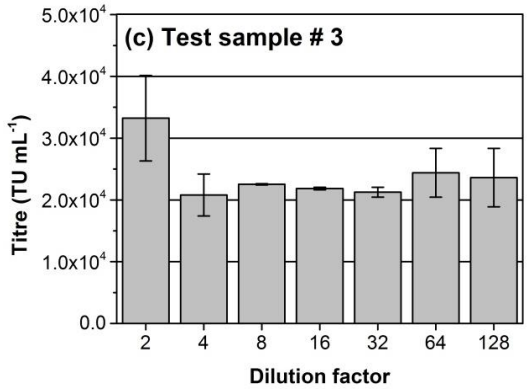
This study relates to Chapter 6, Section 6.2.1.1 of this thesis. In order to define an appropriate lower limit of quantification (LOQ) for the ProSavin® assay, and thus determine a threshold above which titre values may be considered accurate and consequently suitable for inclusion in linear regression analyses, a simple dilution series of lentiviral vector samples was prepared and quantified (Figure B.1). For 2 out of 3 test samples analysed, when a dilution factor of 128 was used (corresponding to a transduced cell population 0.12 - 0.19 %) titres markedly exceeded those recorded for lower dilutions (Figure B.1 [a] and [b]). The data indicates, therefore, that when a standard population size of 10,000 cells is analysed using flow cytometry, accurate quantitation may be expected only when the proportion of transduced cells equals or exceeds around 0.2 %. This threshold exceeds background fluorescence levels by approximately 8-fold, and was subsequently employed as the LOQ for all stability studies described in Chapter 6 (note that the upper limit of accurate quantitation is 20 %; Section 2.11.2.1).



	% transduced cells						
DF	*2*	*4*	*8*	*16*	*32*	*64*	*128*
#1.1	6.33	3.19	1.07	0.62	0.26	0.06	0.12
#1.2	5.58	2.45	1.41	0.59	0.33	0.17	0.13



	% transduced cells						
DF	*2*	*4*	*8*	*16*	*32*	*64*	*128*
#2.1	5.60	2.29	1.18	0.90	0.23	0.23	0.19
#2.2	5.63	2.18	0.96	0.58	0.34	0.18	0.17



	% transduced cells						
DF	*2*	*4*	*8*	*16*	*32*	*64*	*128*
#3.1	8.16	2.46	1.15	0.56	0.26	0.13	0.06
#3.2	5.35	1.77	1.14	0.55	0.28	0.18	0.09

Figure B.1 Analysis of lentiviral vector test samples that had undergone two-fold serial dilutions in order to ascertain a LOQ for the ProSavin® titre assay (Section 2.11.2.1). Test sample #1 (a) was crude supernatant supplied by Charlotte Parker (Oxford BioMedica) and contained ProSavin® generated by transient transfection of adherent HEK293T cells. Test samples #2 (b) and #3 (c) were from the same batch of ProSavin® generated using standard shake flask cultures of PS46.2 (Section 2.4.1.3), but were exposed to different concentrations of polybrene at the transduction stage of the ProSavin® assay (6 and 8 μg mL^{-1}, respectively). Each sample was analysed in duplicate and mean titre values are presented (error bars represent the range). The proportion of cells expressing ProSavin® (following the analysis of 10,000 events) is also indicated below each graph (DF denotes the dilution factor, and the sample reference is indicated). Note that duplicate negative controls (fluorescent-stained non-transduced HEK293T cells) included in the assay returned values of 0.02 and 0.03 % transduced cells.

Appendix C Effect of medium osmolality on the growth of PS46.2 cells in shake flasks

This data is supplementary to that presented in Chapter 6, Section 6.2.2.3 of this thesis, and discusses the concentration and viability of PS46.2 following the three day shake flask acclimatisation period. Following three days cultivation in shake flasks in the various media (Section 6.2.2.3, Table 6.4), large differences in cell growth and viability were observed (Table C.1). Where the medium had not been supplemented with sugars but had been diluted to the same extent as all other runs where a sugar solution had been added (run 1), cells reached a concentration of 35.4 x 10^5 viable cells mL^{-1}, which was equivalent to that attained in the reference culture (34.6 x 10^5 viable cells mL^{-1}; REF, in which neither the sugar or water content of the medium was adjusted). The viability of these cultures was also high (96.3 %) and similar to that observed in the reference culture (95.2 %). The viable cell concentration and viability of all other cultures was negatively correlated with the total sugar content of the medium. Where the standard culture medium had been supplemented with 100 mM fructose, glucose or sorbitol (runs 2, 3 and 5), the viable cell concentration was reduced to 18.8 – 26.9 x 10^5 viable cells mL^{-1}, although viabilities remained high (91.2 - 94.2 %). Where all three sugar types had been added to a final concentration of 150 mM (centre point runs 9 - 12) the viable cell concentration was reduced further to 7.5 x 10^5 viable cells mL^{-1} and cell viability was also reduced (81.7 %). Combinations of two sugar types added to a final concentration of 200 mM (runs 4, 6 and 7) yielded even lower viable cell concentrations (3.6 - 4.5 x 10^5 viable cells mL^{-1}) and cell viabilities (60.3 - 67.9 %), while the addition of all three sugar types to a final concentration of 300 mM (run 8) produced the poorest outcome of all (viable cell concentration 1.5 x 10^5 viable cells mL^{-1} and viability 20.7 %). It was expected that the osmolality of the medium would increase in line with increasing sugar concentrations, and this trend was observed except in the case of run 8, where all sugar types had been added to a final concentration of 300 mM (Section 6.2.2.3, Table 6.4). This medium had an osmolality of 0.456 osmol kg^{-1}, which was slightly lower than those media containing two sugar types added to a final concentration of 200 mM (runs 4, 6 and 7; osmolality 0.470 - 0.473 osmol kg^{-1}). Although all media were mixed by agitation following supplementation and prior to osmolality measurements, it is possible that this was insufficient to attain a homogenous solution in the case of the medium prepared for run 8, which contained the

greatest concentration of sugars overall, and the osmolality was underestimated as a result. It may be concluded that, overall, the viable cell concentration and viability of shake flask cultures was negatively correlated to increasing sugar concentrations and, broadly, to increasing medium osmolalities.

Table C.1 Viable cell concentration and cell viability of PS46.2 following cultivation in shake flasks in medium supplemented with varying concentrations of fructose, glucose and/or sorbitol. The concentration of each sugar type was as indicated in Section 6.2.2.3, Table 6.4. Shake flasks were seeded with 4 x 10^5 viable cells mL^{-1}, and cell counts were performed three days later. Procedures were as outlined in Section 2.9.3. Duplicate measurements were performed and mean values are presented with the range (n = 1).

Run	Viable cell concentration (x 10^5 cells mL^{-1})	Cell viability (%)
1	35.4 ± 2.0	96.3 ± 0.4
2	24.9 ± 2.4	92.9 ± 0.9
3	18.8 ± 0.5	91.2 ± 0.3
4	3.6 ± 0.2	60.3 ± 0.9
5	26.9 ± 1.4	94.2 ± 1.1
6	4.5 ± 0.2	67.9 ± 0.2
7	3.7 ± 0.2	61.7 ± 2.5
8	1.5 ± 0.2	20.7 ± 1.9
9 -12	7.5 ± 0.2	81.7 ± 0.5
REF	34.6 ± 0.4	95.2 ± 0.1

Appendix D Publication

Publication derived from work presented in this thesis:

Guy, H. M., McCloskey, L., Lye, G. J., Mitrophanous, K. A. and Mukhopadhyay, T. K. (2013). Characterization of Lentiviral Vector Production Using Microwell Suspension Cultures of HEK293T-Derived Producer Cells. *Human Gene Therapy Methods* **24(2)**:125-139.

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